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

UNIVERSIDADE ESTATUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA E BIOTECNOLOGIA

PAOLA DA SILVA BALIN

INFLUÊNCIA DA EXPOSIÇÃO *IN UTERO* E LACTACIONAL AO ANTI-INFLAMATÓRIO IBUPROFENO: REPERCUSSÃO TARDIA EM PARÂMETROS REPRODUTIVOS MASCULINOS, EM RATOS

Botucatu – SP

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Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de Pós-Graduação em Farmacologia e Biotecnologia.

Orientadora: Dra. Arielle Cristina Arena

Coorientador: Dr. José de Anchieta de Castro e Horta Júnior

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Epígrafe

"Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante de meus olhos"

(Isaac Newton)

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Resumo

Os anti-inflamatórios não esteroidais (AINEs), entre eles o Ibuprofeno, são amplamente utilizados para o tratamento da dor e de processos inflamatórios, e estão entre as classes de medicamentos mais utilizadas por gestantes. Através da inibição da enzima ciclo-oxigenase, os AINEs inibem a síntese de prostaglandinas, compostos eicosanoides que atuam não somente como mediadores e moduladores inflamatórios, mas também em diversos processos fisiológicos do organismo, como no mecanismo de diferenciação sexual hipotalâmica. O processo de masculinização do hipotálamo é dependente de testosterona, que por ação da enzima citocromo P450 aromatase, é convertida em estradiol. Este hormônio regula positivamente a expressão da enzima ciclo-oxigenase no hipotálamo, aumentando a produção de prostaglandina do subtipo E_2 (PGE_2), que atua aumentando a formação de espinhas dendríticas no núcleo sexualmente dimórfico da área pré-optica (SDN-POA). Em virtude da importância da PGE_2 no processo de diferenciação sexual hipotalâmica, torna-se preocupante o uso de anti-inflamatórios durante a gestação. Desta forma, o objetivo desse estudo foi avaliar os possíveis efeitos resultantes da exposição *in utero* e lactacional ao ibuprofeno e suas repercussões tardias sobre parâmetros reprodutivos masculinos em ratos machos. Para tanto, ratas prenhes foram expostas a três doses de ibuprofeno (10; 30; 60 mg/kg) entre a última semana de prenhez (Dias gestacionais 15-21) até o final da lactação (Dias pós-natal 21) por gavage. Durante o tratamento, foram monitorados o consumo de água e ração, massa corporal e comportamento materno das ratas expostas. Após o desmame, coletou-se o sangue das mães para análises bioquímicas e órgãos para registro do peso. Após o nascimento, os filhotes machos foram avaliados através dos seguintes parâmetros: massa corporal, distância anogenital e idades de descida testicular e separação prepucial. Na vida adulta, estes mesmos animais foram investigados em relação a parâmetros comportamentais (comportamento sexual masculino e feminino e preferência sexual), fertilidade,

parâmetros espermáticos (contagem, motilidade e morfologia espermática), pesos de órgãos reprodutores, dosagem hormonal, quantificação do volume do SDN-POA e histologia de testículo e epidídimo. O tratamento com ibuprofeno não alterou os parâmetros maternos avaliados. A prole masculina apresentou redução na massa corporal e na distância anogenital, bem como, atraso nas idades de descida testicular e separação prepucial. Na vida adulta, os animais apresentaram redução nos níveis séricos de testosterona e no volume do núcleo de célula de Leydig. A exposição ao ibuprofeno não alterou os pesos dos órgãos reprodutores, a contagem espermática, a fertilidade ou o número de células de Sertoli, entretanto causou diminuição no número de espermatozoides normais. Em relação aos parâmetros comportamentais, os animais expostos ao ibuprofeno apresentaram tanto comportamento sexual masculino quanto feminino, contudo não houve alteração em relação ao padrão de preferência sexual. O volume total do SDN-POA não foi alterado. Concluiu-se que o ibuprofeno, nestas condições experimentais, foi capaz de perturbar a programação do eixo hipotálamo-hipófise-gônada, afetou a maturação sexual e as funções reprodutivas masculinas.

Palavras chaves: Prostaglandinas, diferenciação sexual hipotalâmica, área pré-óptica, núcleo sexualmente dimórfico, comportamento sexual.

Abstract

Non-steroidal anti-inflammatory drugs (NSAID), including Ibuprofen, are widely used in the treatment of pain and inflammatory processes, and are of the most commonly classes of drugs used by pregnant women. By inhibiting the cyclooxygenase enzyme (COX), NSAID inhibit the synthesis of prostaglandins, eicosanoids compounds that act not only as mediators and inflammatory modulators, but also in various physiological processes of the organism, such as in the mechanism of sexual hypothalamic differentiation. The hypothalamus masculinization process is testosterone dependent, which by action of the aromatase cytochrome P450 enzyme is metabolized to estradiol. This hormone upregulates the expression of COX enzyme in the hypothalamus, increasing the production of prostaglandin E_2, which acts by increasing the formation of dendritic spines in the neurons of the sexually dimorphic nucleus of the preoptic area in males (SDN-POA). Due to the importance of prostaglandin E_2 in the process of hypothalamic sexual differentiation, the use of anti-inflammatory drugs during pregnancy is of concern. Thus, the aim of this study was to evaluate the possible effects resulting from *in utero* and lactation exposure to non-steroidal anti-inflammatory ibuprofen and its late repercussions on male reproductive parameters in male rats. For this, pregnant rats were exposed to three doses of ibuprofen (10; 30; 60 mg/kg) between the last week of pregnancy (Gestational Days 15-21) until the end of lactation (Postnatal Days 21) by gavage. During treatment, water consumption, food intake, body weight and maternal behavior of the dams were monitored. After weaning, blood samples was collected for biochemical analyzes and organs for weight recording. After birth, male offspring were evaluated through the following parameters: body weight, anogenital distance and ages of testicular descent and preputial separation. In the adult life, these same animals were investigated in relation to behavioral parameters (male and female sexual behavior and sexual preference), fertility, sperm parameters (sperm count, sperm

motility and sperm morphology), reproductive organ weights, hormonal levels, volume of the SDN-POA and histology of testis and epididymis. Treatment did not affect any of the maternal parameters evaluated. Male offspring presented reduction in body weight and anogenital distance, as well as delay in the ages of testicular descent and preputial separation. In adulthood, these animals showed reduced serum testosterone levels and the volume of Leydig cell nucleus. Exposure to ibuprofen did not alter reproductive organ weights, sperm count, fertility or the number of Sertoli cells; however it resulted in a decrease in the number of normal sperm. Regarding the behavioral parameters, the animals exposed to ibuprofen presented both male and female sexual behavior, but there was no change in relation to the pattern of sexual preference. The total volume of the sexually dimorphic nucleus of the preoptic area was not altered. It was concluded that ibuprofen, under these experimental conditions, was able to disturb the hypothalamic-pituitary-gonadal axis programming, affected sexual maturation and male reproductive functions.

Key words: Prostaglandins, hypothalamic sexual differentiation, preoptic area, sexually dimorphic nucleus, sexual behavior.

Introdução

1. Diferenciação Sexual Hipotalâmica

Cérebro e gônadas são órgãos bipotenciais durante o desenvolvimento embrionário, e as sinalizações hormonais são responsáveis por diferenciá-los em femininos ou masculinos (Schwarz & McCarthy, 2008). Assim, no início do desenvolvimento, os mamíferos precisam passar pelo processo de determinação sexual, em que o sexo genético (cromossômico) determinará o sexo gonadal, e de diferenciação sexual, no qual os hormônios gonadais determinarão o sexo do cérebro (McCarthy & Arnold, 2011).

Em machos (XY), o desenvolvimento dos testículos está condicionado a presença do gene SRY, localizado no braço curto do cromossomo Y, responsável pela síntese do fator determinante de testículos (Koopman et at., 1990). Em fêmeas (XX), a ausência desse gene faz com que a gônada bipotente torne-se um ovário (Sinclair et al., 1990). Depois de desenvolvidos, os testículos começam a produzir quantidades significativas de testosterona (Weisz & Ward, 1980; Rhoda et al., 1984), a qual induzirá a formação do epidídimo, ducto deferente, glândulas sexuais acessórias e pênis (Jost, 1947). Em um determinado momento, no encéfalo a testosterona é convertida a estradiol, responsável por induzir mudanças em regiões hipotalâmicas, determinando assim a diferenciação sexual do cérebro, garantindo que o sexo gonadal seja o mesmo do cérebro (Figura 1) (Schwarz & McCarthy, 2008).

Antes do período crítico de diferenciação sexual hipotalâmica, definido como uma janela em que o cérebro esta particularmente sensível a ação hormonal, o hipotálamo de mamíferos está organizado intrinsecamente do tipo feminino, o que irá determinar na vida adulta, um comportamento sexual típico de fêmea e um padrão cíclico de secreção gonadotrofinas. Nos machos, para que ocorra o comportamento sexual tipicamente masculino e apareça o padrão tônico de secreção de gonadotrofinas,

o hipotálamo precisa ser masculinizado (Maclusky & Naftolin, 1981). Sendo assim, a diferenciação do sistema nervoso central (SNC) em machos é determinada por dois processos distintos: a defeminização e a masculinização. Em modelos experimentais, o processo de defeminização compreende a indução de esterilidade anovulatória e a redução da capacidade de apresentar lordose, já a masculinização é definida como a capacidade de apresentar comportamento de monta (McEwen, 1977).

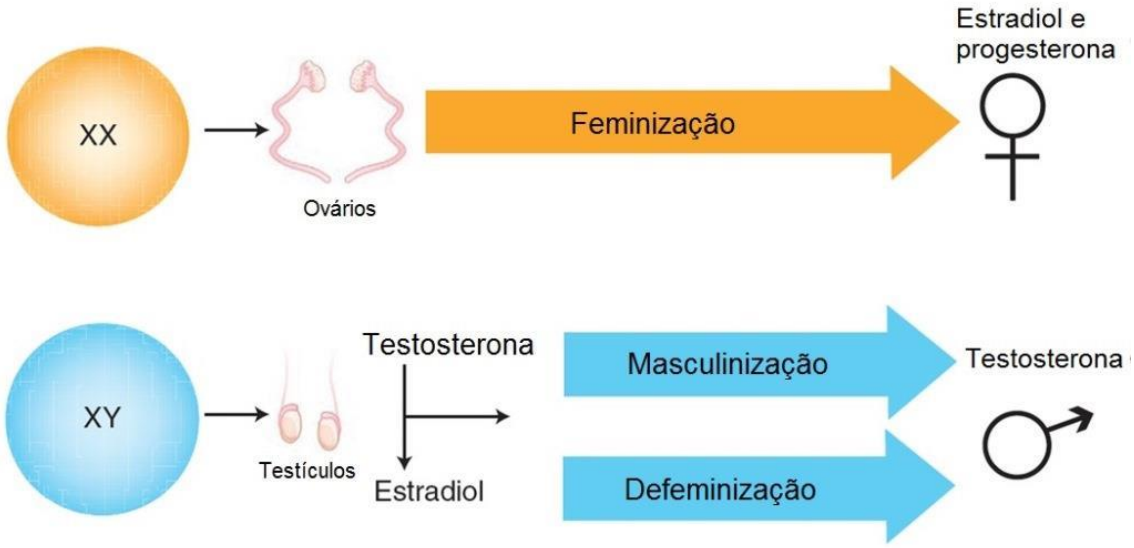

Figura 1- Visão linear do processo de diferenciação sexual, em que o sexo cromossômico determina sexo gonadal, que determina o sexo cerebral (Adaptado de McCarthy & Arnold, 2011).

O processo de masculinização do hipotálamo é dependente de testosterona, que por ação da enzima citocromo P450 aromatase, é metabolizada originando estrógeno no SNC (Rhoda et al., 1984; Erskine et al., 1988). O estrógeno liga-se a dois subtipos de receptores, o receptor de estrógeno α, envolvido com o processo de masculinização, e o receptor β, que tem maior função no processo de defeminização (Kudwa et al., 2006).

O momento exato do processo de masculinização cerebral em humanos ainda não está completamente elucidado (McCarthy et al., 2009). Sabe-se que a primeira "onda" de testosterona proveniente da gônada masculina ocorre entre a sexta e oitava

semanas de gestação e é responsável pela diferenciação da genitália (Auyeung et al., 2009). Uma segunda "onda" de esteroides sexuais ocorre na puberdade, sendo considerada uma fase de "refinamento" da diferenciação sexual e nesse momento, ocorre a organização dos circuitos neurais sexuais no cérebro do adolescente em desenvolvimento, facilitando a expressão do comportamento sexual (Schulz et al., 2009). Já em ratos, a elevação dos níveis de testosterona gonadal, relacionados ao mecanismo de diferenciação sexual hipotalâmica, ocorre no final da gestação e nos dez primeiros dias de vida pós-natal, apresentando dois picos, o primeiro no 18º e 19º dia de gestação (Weisz & Ward, 1980; McCarthy et al., 2009), e o segundo durante as primeiras horas após o nascimento (Corbier et al., 1978; Baum et al., 1988; Lalau et al., 1990; McCarthy et al., 2009). Corbier et al. (1992), em estudo comparativo, demonstraram que ocorre também uma elevação nos níveis de testosterona, após as primeiras horas do nascimento, numa variedade de mamíferos machos, como camundongos, cavalos e humanos, corroborando a importância dos andrógenos na diferenciação sexual do SNC desta classe de vertebrados.

Evidências sugerem que a conversão de testosterona a estradiol pela aromatase neural é o mecanismo responsável por regular diversos processos fisiológicos e comportamentais, como a ativação do comportamento sexual masculino, a diferenciação sexual hipotalâmica e efeitos na retroalimentação negativa sobre a secreção de gonadotrofinas (Balthazart & Ball, 1998). Dessa forma, substâncias capazes de suprimir ou retardar o pico de testosterona neonatal podem alterar o processo de masculinização e/ou defeminização do hipotálamo (Gore, 2010).

Para garantir o dimorfismo sexual cerebral, a aromatização da testosterona é um evento necessário, e o estrógeno resultante é o responsável por atuar nos núcleos hipotalâmicos para garantir a masculinização do SNC de roedores (Naftolin et al.,

1975). Através de mecanismos distintos, o estradiol induz mudanças permanentes em diferentes regiões do cérebro durante o período crítico de desenvolvimento (Figura 2). Alguns dos mecanismos relacionados à masculinização do hipotálamo são: indução de apoptose celular, gerando diferenças sexuais volumétricas nos núcleos hipotalâmicos; alteração da função neurotransmissora no hipotálamo mediobasal; aumento na expressão de enzimas envolvidas na síntese do ácido gama-aminobutírico (GABA), aumentando a complexidade dos astrócitos no núcleo arqueado de machos, e também a indução de diferença sexual morfológica e volumétrica nos neurônios e astrócitos do núcleo sexualmente dimórfico da área pré-optica (SDN-POA) via prostaglandina E_2 (PGE_2), sendo esta via prostanoide a mais importante relacionada ao processo de masculinização e defeminização de machos (Schwarz & McCarthy, 2008).

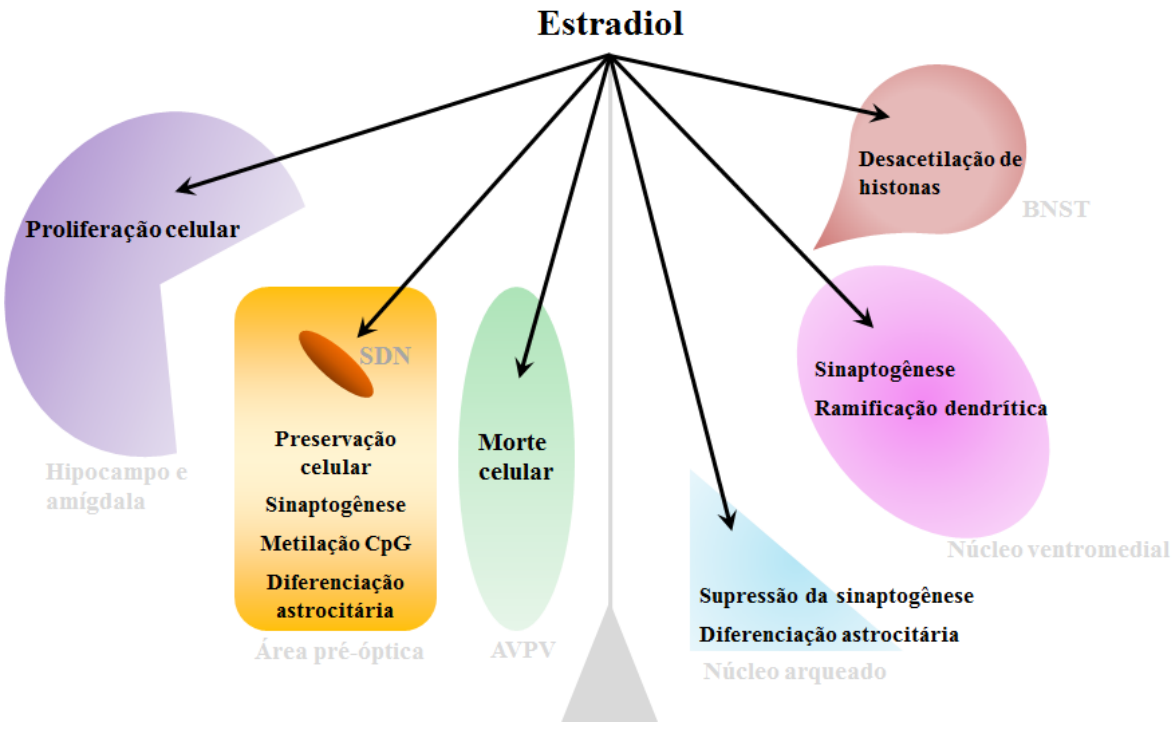

Figura 2 - Múltiplos mecanismos de diferenciação induzidos pelo estradiol (Adaptado de McCarthy & Arnold, 2011).

Existem vários tipos conhecidos de prostaglandinas (PG), com subtipos e padrões de receptores distribuídos no organismo. As prostaglandinas são compostos

eicosanoides que atuam não somente como mediadores e moduladores inflamatórios, mas também estão implicados em diversos processos fisiológicos do organismo (Rang et al., 2012; Smyth et al., 2012), como por exemplo, no mecanismo de diferenciação sexual hipotalâmica (Amateu & McCarthy, 2004). Uma evidência de que a PGE_2 é um regulador potente do desenvolvimento normal do sexo masculino foi demonstrada por Todd et al. (2005). Neste estudo, ratos machos tratados ao nascer com Indometacina, um anti-inflmatório não esteroidal (AINE) inibidor seletivo da ciclo-oxigenase 2 (COX-2) (enzima responsável pela conversão de ácido araquidônico em prostaglandinas), não exibiram comportamento sexual masculino ou feminino na idade adulta, indicando que eles foram completamente defeminizados, mas não masculinizados, demonstrando o papel chave da PGE_2 no processo de masculinização do hipotálamo mediado pelo estradiol.

Enquanto o processo de diferenciação sexual em machos é bastante estudado e conhecido, o mesmo nas fêmeas ainda é pouco elucidado. Por muito tempo acreditou-se que o processo de feminização do cérebro feminino ocorria de forma passiva, na ausência de qualquer secreção hormonal. Entretanto, este conceito vem sendo contestado, pois há indícios de que este também seja um processo ativo dependente de estradiol (Toran-Allerand, 1976; Döhleret et al., 1984; Mack et al., 1993). Sabe-se que a alfa-fetoproteína (AFP), uma glicoproteína plasmática produzida em grandes quantidades durante a vida fetal pelas células da endoderme do saco vitelino, pelos hepatócitos e, em menor quantidade, pelo trato gastrointestinal, protege o cérebro fetal feminino da exposição a estrógenos circulantes maternos, pois se liga com elevada afinidade ao estrógeno não permitindo que este atinja o hipotálamo. A síntese de AFP diminui acentuadamente logo após o nascimento e apenas quantidades traços são detectadas em adultos (Bakker et al., 2006; Bakker & Baum, 2008).

Contudo, os dados existentes na literatura não identificaram até o momento em qual estágio do desenvolvimento (embrionário, pós-natal, pré-pubere ou pós-púbere), ocorre a janela crítica para diferenciação sexual hipotalâmica em fêmeas.

Devido ao fato do processo de diferenciação sexual hipotalâmica ser controlado por diferentes mecanismos, estudos demonstram que o estresse, a exposição a contaminantes ambientais, bem como a administração de medicamentos durante seu período crítico podem comprometer esse processo, acarretando em alterações na fisiologia reprodutiva e no comportamento sexual (Arena & Pereira, 2002; Pereira & Piffer, 2005; Negri-Cesi, 2015), as quais muitas vezes somente serão detectadas mais tarde, na vida adulta reprodutiva.

1.1 Núcleo Sexualmente Dimórfico da Área Pré-óptica (SDN-POA)

O hipotálamo, pequena área cerebral localizada abaixo da comissura anterior, está envolvido na regulação de diversas funções, incluindo a reprodução (Gore, 2010). Esta área contém diversos núcleos sexualmente dimórficos, dentre eles, o SDN-POA, um núcleo ovoide, com células densamente agrupadas, localizado na porção medial da área pré-óptica hipotalâmica, caracterizado por sua coloração mais intensa e por possuir corpos celulares maiores em comparação com a área pré-ótica circundante (Figura 3A) (Hofman & Swaab, 1989). Pode ser definido pela sua localização em relação a vários marcos anatômicos, incluindo o 3º ventrículo, quiasma óptico e a comissura anterior (Figuras 3B-C).

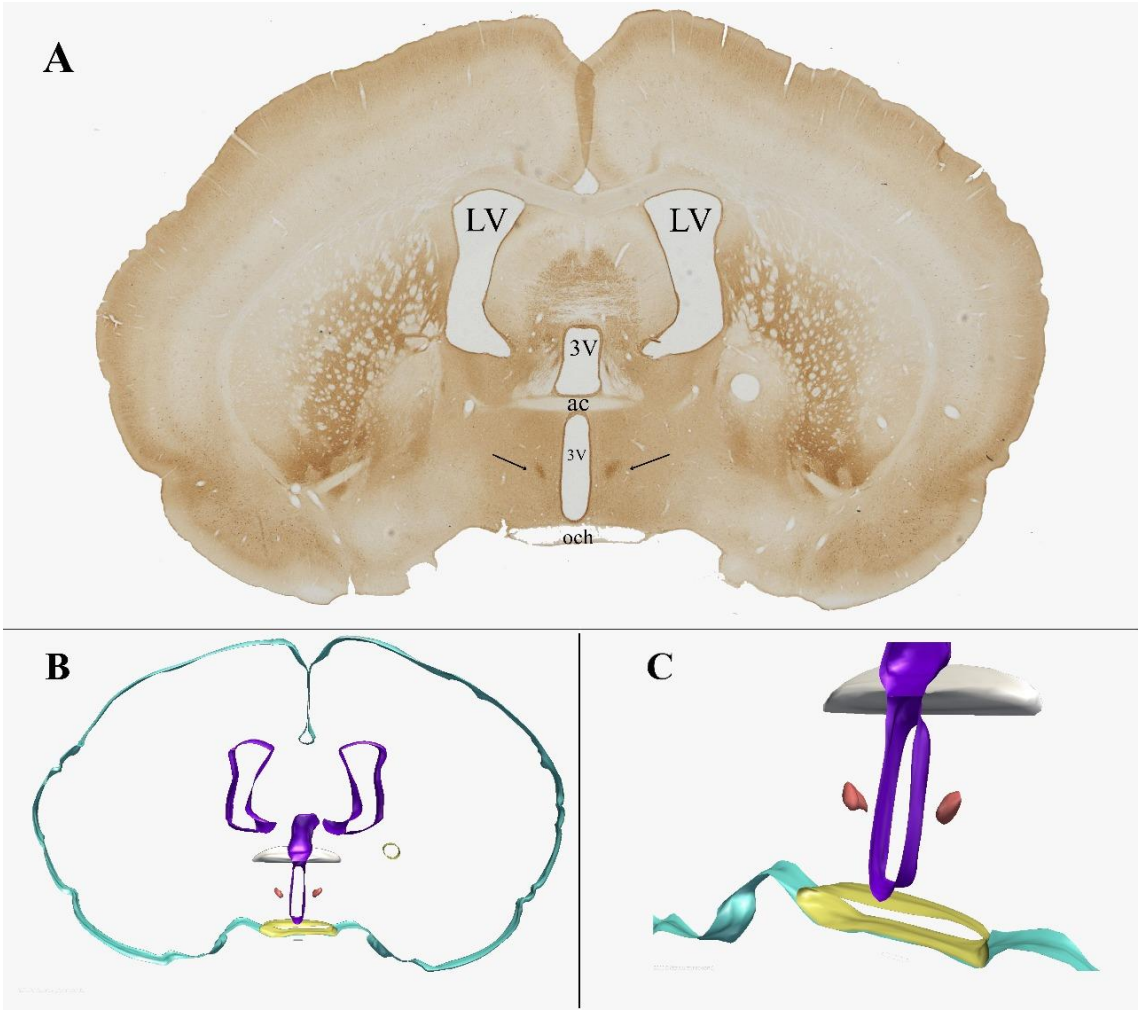

Figura 3 – Em A: Secção coronal do encéfalo de rato, onde as setas apontam para o SDN-POA marcado através de técnica de imuno-histoquímica para Calbindina D-28K. LV: ventrículo lateral, 3V: 3º ventrículo, ac: comissura anterior, och: quiasma óptico. Em B e C Modelos tridimensionais em secção coronal. Contorno externo do encéfalo (azul-claro), ventrículos laterais e 3º ventrículo (roxo), comissura anterior (branco), quiasma óptico (amarelo) e SDN-POA (vermelho). (Fonte: o autor)

O SDN-POA é a porção do hipotálamo que faz parte do circuito neural subjacente aos processos reprodutivos masculinos, o responsável pela mediação e regulação do comportamento sexual masculino e pelas funções reprodutivas endocrinológicas dos machos (Amateau & McCarthy, 2002). O núcleo intersticial do hipotálamo anterior 3 (INAH3) no ser humano é considerado como o equivalente estrutural do SDN-POA do rato (Garcia-Falgueras & Swaab, 2008).

Durante o período crítico de diferenciação sexual hipotalâmica dos machos, o estrógeno, resultante da aromatização da testosterona, regula positivamente a expressão da enzima COX-2, e consequentemente aumenta a produção de prostaglandina do subtipo E_2 (McCarthy, 2008). Este prostanoide é liberado pelos neurônios e age em astrócitos vizinhos, levando-os a liberar glutamato (Bezzi et al., 1998) que, por sua vez, atua induzindo a formação de espinhas dendríticas no cérebro de roedores (Figura 4) (Amateau & McCarthy, 2002). Um macho recém-nascido apresenta de 2 a 3 vezes mais receptores de estrógeno do que o hipotálamo de fêmeas, e o SDN-POA é de 3 a 5 vezes maior em machos (McCarthy, 2008).

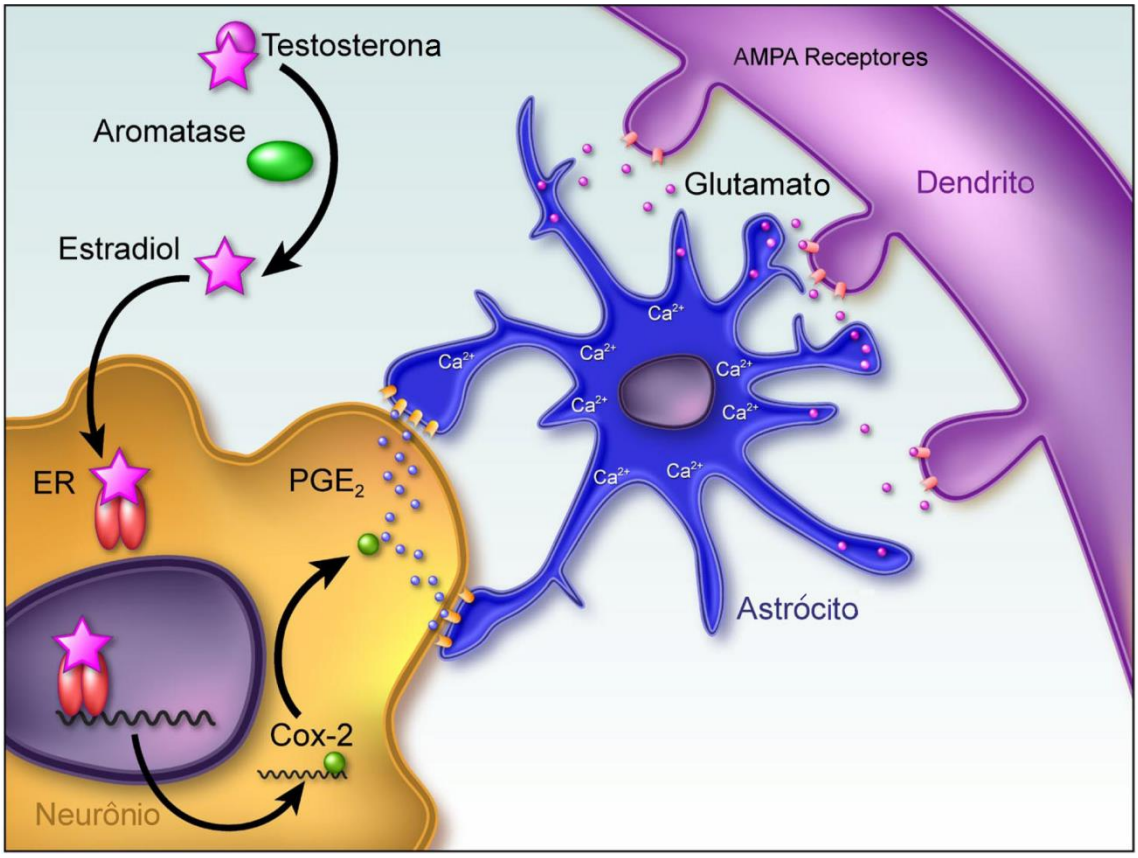

Figura 4 - Mecanismo de ação do estradiol pela via prostanoide no núcleo sexualmente dimórfico na área pré-óptica (Adaptado de McCarthy, 2008).

Desta forma, substâncias capazes de afetar os picos de testosterona fetal ou alterar a expressão de receptores e enzimas envolvidas na diferenciação sexual hipotalâmica, podem alterar o processo de masculinização e/ou defeminização do hipotálamo (Gore, 2010).

2. Problemática do uso de medicamentos na gestação e lactação

A administração de medicamentos, por prescrição ou por automedicação, durante o período gestacional é alta. Cerca de 40 a 90% das gestantes utilizam pelo menos uma substância durante este período, excluindo-se os polivitamínicos (Carvalho et al., 2012). Porém, esta é uma prática que requer atenção especial, devido ao risco de desfechos adversos da gestação.

No início da década de 1960, o episódio que ficou conhecido como a tragédia da talidomida, no qual cerca de 10 mil crianças nasceram com malformações congênitas, serviu de alerta internacional quanto à segurança do uso de medicamentos durante a gestação (Febrasco, 2011). Até então, acreditava-se que a placenta funcionava como uma "barreira" que protegia o feto de qualquer agente químico, porém, atualmente sabe-se que diversas moléculas podem cruzar essa membrana livremente e chegar à circulação fetal (Schimmer & Parker, 2012). Vale ressaltar que as lactantes também se enquadram em uma situação especial, visto que alguns fármacos são secretados no leite materno, podendo expor o lactente a níveis tóxicos durante o período pós-natal, fase em que o neonato ainda esta em desenvolvimento (Ito & Lee, 2003).

Antes de serem aprovadas para comercialização, novas substâncias farmacologicamente ativas devem passar por uma série de ensaios clínicos projetados para estabelecer sua segurança e eficácia (CNS, 1997). Inicialmente, os testes são realizados em um grupo reduzido de humanos saudáveis (fase I); depois em um grupo

de pacientes (fase II); seguido por um ensaio multicêntrico com pacientes (fase III); e por fim o período de vigilância pós-comercialização (fase IV) (FDA, 1977; CNS, 1997; Rivera & Gilman, 2012). Entretanto, não é considerado eticamente aceitável realizar estudos clínicos pré-comercialização em gestantes, sendo assim, grande parte das informações sobre a segurança dos medicamentos nessa população são provenientes de relatos de caso, de estudos retrospectivos não controlados ou de estudos de teratogenicidade e toxicidade reprodutiva realizados em modelos animais (CNS, 1996; Carvalho et al., 2012). Atualmente, graças a esses estudos, diversas substâncias já tem seu potencial teratogênico conhecido, e a partir disso o *Food and Drug Administration* (FDA) classifica as drogas em categorias (A, B, C, D e X), de acordo com o seu risco na gestação (FDA, 1977; Schimmer & Parker, 2012).

Segundo o FDA, os AINE, como o ibuprofeno, cetoprofeno e naproxeno, são classificados como categoria de risco B na gestação, entretanto o FDA recomenda que o uso desses fármacos seja evitado no terceiro trimestre, já que esses medicamentos podem provocar o fechamento precoce do ducto arterioso (Carvalho et al., 2012). Contudo, estudos apontam que se deve ter cautela na administração deste grupo farmacológico também durante os dois primeiros trimestres de gestação, pois seu uso parece estar associado à criptorquidia congênita e ao aumento nas taxas de aborto espontâneo (Jensen et al., 2010; Bloor & Paech, 2013).

Mengue e colaboradores (2001), analisaram o consumo de medicamentos por gestantes em 6 cidades brasileiras (São Paulo, Rio de Janeiro, Porto Alegre, Salvador, Fortaleza e Manaus), e constataram que os AINE são a terceira classe de medicamentos mais utilizadas por gestantes. Em um estudo multicêntrico realizado em 22 países pela *Collaborative Group on Drug Use in Pregnancy*, financiado pela Organização Mundial da Saúde (OMS), os analgésicos e anti-inflamatórios apareceram como a terceira classe

medicamentosa mais prescrita durante a gestação, e a primeira classe mais utilizada no cenário da automedicação (CGDUP, 1992).

Em virtude do alto consumo de AINE por gestantes e da importância da PGE_2 no processo de diferenciação sexual hipotalâmica, torna-se preocupante o uso de anti-inflamatórios durante a gestação.

3. Ibuprofeno

O ibuprofeno ((*RS*)-2-(4-(2-metilpropil) fenil) ácido propanoico) (Figura 5), fórmula molecular $C_{13}H_{18}O_2$, apresenta-se como um pó branco ou quase branco, com odor característico (ANVISA, 2010). É um AINE derivado do ácido propiônico, inibidor não seletivo da enzima ciclo-oxigenase (COX-1 e COX-2), reduzindo assim, a produção de prostaglandinas (PG). Os AINEs estão entre os medicamentos mais comuns prescritos para mulheres grávidas para tratar febre, dor e inflamação (Østensen & Skomsvoll, 2004).

Esse fármaco possui rápida absorção no trato gastrointestinal. Após administração oral, 80% da dose é absorvida, apresenta alto potencial de ligação com proteínas (cerca de 99%), com meia vida de aproximadamente 2h. Sofre metabolismo hepático e a excreção dos metabólitos ocorre por via renal. Pertence à Classe II do Sistema de Classificação Biofarmacêutico (BCS), apresentando baixa solubilidade aquosa e elevada permeabilidade membranar. Tanto o ibuprofeno quanto seus metabólitos atravessam a placenta. (Grosser et al., 2012)

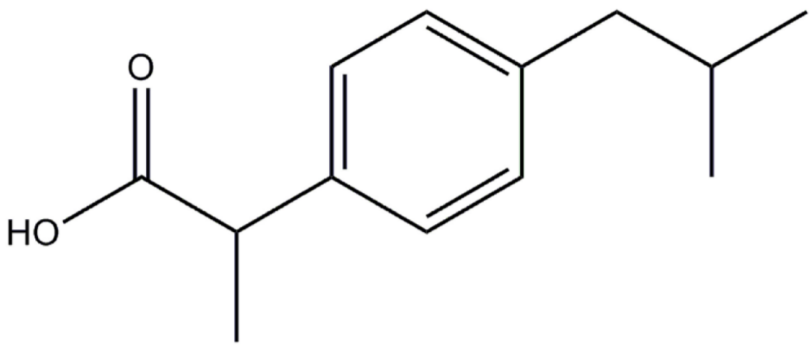

Figura 5 - Fórmula estrutural do ibuprofeno.

O ibuprofeno é considerado um medicamento isento de prescrição (MIP), também chamados de over-the-counter (OTC), comercializado na forma de comprimido, pó, granulado, suspensão oral, cápsula gelatinosa e supositório, em doses que variam entre 50 e 800 mg, sendo a dose diária máxima permitida em adultos de 3200mg. A dose letal$_{50}$ (DL_{50}) por via oral avaliada é de 636 mg/kg em ratos e 740 mg/kg em camundongos (Lewis, 1996).

Por ser amplamente utilizado em doses elevadas, a probabilidade de ocorrência deste fármaco no ambiente aumenta, decorrente da excreção humana. Alguns estudos relataram a presença de ibuprofeno em águas superficiais e efluentes ao redor do mundo (Alemanha, Reino Unido, Finlândia, França, Suíça e EUA), sendo que em alguns casos, o ibuprofeno resiste ao processo de tratamento de água, atingindo a água de consumo (Ternes, 1998; Heberer., 2002; Hilton & Thomas, 2003; Ashton et al., 2004; Robert & Thomas, 2006; Skadsen et al., 2006; Vieno et al., 2007; Togola & Budzinski, 2008; Mompelat et al., 2009).

Em virtude do que foi exposto, torna-se necessário avaliar os efeitos da exposição a essa classe de fármacos durante períodos críticos do desenvolvimento (*in utero* e lactação), visto que, alterações em processos que ocorrem durante esse período geralmente serão percebidas somente na puberdade ou na vida adulta reprodutiva

(Pereira, 2003; Pereira & Piffer, 2005). Desta forma, através do modelo experimental proposto neste trabalho, procura-se evidenciar possíveis interferências da administração de ibuprofeno durante o período gestacional e lactacional, na prole masculina.

Justificativa

A utilização dos AINEs, como o ibuprofeno, por gestantes é uma realidade mundial, e a exposição a essas substâncias inibidoras da enzima COX pode interferir no processo de diferenciação sexual da prole masculina, sendo que as repercussões somente serão notadas na vida adulta reprodutiva.

Ademais, estudos recentes reportam que AINEs, podem ser considerados desreguladores endócrinos com efeitos antiandrogênicos, pois afetam o processo de diferenciação sexual em humanos e ratos (Kristensen et al., 2011a,b; 2012). Este efeito reforça a hipótese que deve haver conexões intrínsecas desconhecidas entre as PGs e os andrógenos.

Desse modo, os resultados obtidos no presente estudo podem fornecer informações importantes para a implementação de políticas governamentais de saúde pública que visem um controle mais eficaz sobre a utilização dessa classe de medicamentos na gestação.

Objetivo

Objetivo Geral

Avaliar os efeitos resultantes da exposição ao ibuprofeno durante o período crítico de diferenciação sexual masculino e as possíveis repercussões tardias sobre o comportamento sexual e a qualidade espermática em ratos machos adultos.

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Capítulo 1

O presente trabalho deu origem ao manuscrito intitulado "**Maternal exposure to ibuprofen can affect the programming of the hypothalamus of the male offspring"** que será submetido à revista Hormones and Behavior, ISSN: 0018-506X, Fator de impacto: 3,378

Maternal exposure to ibuprofen can affect the programming of the hypothalamus of the male offspring

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Highlights

- Ibuprofen exposure decreased the body weight and anogenital distance of the offspring.
- There was a delay in puberty installation.
- There was a decrease in the volume of Leydig cells nucleus and testosterone levels were reduced.
- Sexual behavior in adult life was affected.

Abstract

Ibuprofen, a non-steroidal anti-inflammatory drug, act through inhibition of the cyclooxygenase enzyme, leading to Prostaglandin E_2 (PGE_2) reduction. Due to the importance of PGE_2 to promote the masculinization of hypothalamus during the process of hypothalamic sexual differentiation, the use of anti-inflammatory drugs during pregnancy is of concern. The aim of this study was evaluated the effects of *in utero* and lactational exposure to ibuprofen and its late repercussions on reproductive parameters in male rats. Pregnant rats were exposed to ibuprofen (10; 30 and 60 mg/kg) per gavage between the last week of pregnancy until the end of lactation. After birth, the male offspring were evaluated using the initial developmental parameters and installation of puberty. In adult life, the following parameters were evaluated: organs weight, sperm count, motility and morphology, testosterone levels, histological analysis of reproductive organs, sexual behavior, sexual preference, fertility test and volume of the sexually dimorphic nucleus of the preoptic area. Male offspring exposed to ibuprofen had a decrease in body weight and in the anogenital distance. A delay in the ages of testicular descent and in the preputial separation was also observed. In adulthood, there was a decrease in the Leydig cells volume, in the testosterone levels and in the number of normal sperm morphology. The animals exposed to ibuprofen presented male sexual behavior, however, in the presence of another male, they also presented a female behavior. Maternal exposure to ibuprofen during the critical windows of development adversely impact hypothalamus–pituitary–gonadal axis, sexual maturation and reproductive function.

Key words: Prostaglandins, hypothalamic sexual differentiation, preoptic area, sexually dimorphic nucleus, sexual behavior.

Introduction

Epidemiological studies show that nonsteroidal anti-inflammatory drugs (NSAID) are one of the most frequently consumed drugs during pregnancy (CGDUP, 1992; Olsen et al., 2001; Kozer et al., 2003; Kristensen et al., 2016). These drugs have been prescribed to treat inflammatory processes as well as isolated pain and fever. The risk of using this class of drugs during this critical period of development is the high membrane permeability, allowing freely crossing the placenta reaching the fetus (Siu et al., 2000; Alano et al., 2001).

Furthermore, pharmaceuticals are mainly eliminated in the urine, either in their original form or as metabolites. Therefore, medications like paracetamol, acetylsalicylic acid and ibuprofen are at the top of the list of pharmaceuticals identified in sewage treatment effluents, and in some cases, resisting the water treatment process, reaching drinking water (Cahill et al., 2004; Bouissou-Schurtz et al., 2014). Thus, pregnant and lactating women and even children are at risk of being exposed indirectly to NSAID.

Ibuprofen is a NSAID derived from propionic acid, it is considered an over-the-counter drug and acts by inhibiting the two isoforms of the cyclooxygenase enzyme, COX-1 and COX-2, leading to prostaglandin E_2 (PGE_2) reduction. The PGE_2 are eicosanoids compounds which act not only as mediators and inflammatory modulators, but are also implicated in various physiological processes of the organism (Rang et al., 2012; Smyth et al., 2012), as for example in the process of hypothalamic sexual differentiation (Amateu and McCarthy, 2004).

The neonatal brain, especially the hypothalamus, is influenced by steroid hormones of gonadal origin to produce sex differences underlying sexual behavior (Lenz and McCarthy, 2010). Thus, a male-phenotypic brain requires two active processes during its development: masculinization and defeminization. Masculinization

is the organization of a neural substrate permissive to the adult expression of male sexual behavior, while the defeminization is the loss of capacity as an adult to respond to activational effects of estradiol and progesterone to induce female sexual behaviors (Baum, 1979; Nordeen and Yahr, 1983).

The process of sexual differentiation of the brain in males is initiated by testicular synthesis of testosterone; in rats occurs during the last week of gestation until the first ten days postnatally, with two important peaks, the first between gestational days (GD) 18 and 19 and the second in the first 2 hours after birth. The fetal testosterone gains access to the brain and is converted to its end product, estradiol (E_2), by the p450 aromatase enzyme, which acts in the sexually dimorphic nucleus of preoptic area (SDN-POA) (Naftolin et al., 1975; McEwen et al., 1977; Wersinger et al., 1997), portion of the hypothalamus responsible for the mediation and regulation of male sexual behavior and the endocrine reproductive functions of males (Amateu and McCarthy, 2002).

The estradiol, resulting from testosterone aromatization, upregulate the expression of COX-2 enzyme and increase prostaglandin production, that is released by neurons acts on astrocytes, leading them to release glutamate, which induces the formation of dendritic spines in the male brain, making the SDN-POA 3 to 5 times higher in males (Bezzi et al., 1998; Amateau and McCarthy, 2002; McCarthy, 2008).

PGE_2 is a mediator as important as estradiol (E_2) in the process of formation of SDN-POA. Cultured POA neurons exposed to either E_2 or PGE_2 exhibited an almost three fold rise in the number and density of dendritic spines, while the effects of E_2 were blocked by the prostanoid synthesis inhibitor, indomethacin, demonstrating that the PGE_2 is necessary and sufficient to masculinize the morphology of POA (Amateau and McCarthy, 2002). Another evidence that PGE_2 is a potent regulator of normal male

development has been demonstrated by Todd et al. (2005), male neonate rats treated with COX-2 inhibitors displayed neither male nor female sexual behavior as adults; they were asexual. Conversely, females treated with PGE_2 displayed male sexual behavior as adults.

Therefore, substances able to inhibit PG synthesis, such as ibuprofen, during critical periods of development may have consequences similar to an inhibitor of testosterone or estradiol. The aim of this study was to evaluate the consequences of maternal exposure to ibuprofen during pregnancy and lactation on the sexual differentiation process, sexual maturation and reproductive function in the male offspring.

Materials and Methods

Animals

Forty adult male (90 days old/ 350-400 g) and 50 female (60 days old/ 200 g) Wistar rats were obtained from the Central Biotherium of São Paulo State University (UNESP) and maintained under controlled conditions (23°C, 12/12-h light/dark cycle) with food and water available *ad libitum*. One nulliparous female rat was mated with one male, during the dark cycle of the photoperiod. The detection of sperm in the vaginal smear of rats in oestrus was considered as gestational day 0 (GD0). Pregnant and lactating rats were maintained in individual cages.

The experimental procedures were in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and was approved by the Ethics Committee for Animal Experimentation at the Institute of Biosciences of Botucatu/UNESP (Protocol number: 830/2016).

Experimental groups and treatment

Pregnant rats were distributed into four experimental groups: control group (n= 11, treated with corn oil (vehicle) alone) and three groups treated with ibuprofen ((RS)-2-(4-(2-methylpropyl)phenyl)propanoic acid, Shandong Xinhua Pharmaceutical Co., Ltd. China, 99,65% purity) at doses of 10 mg/kg (n=12); 30 mg/kg (n=11) or 60 mg/kg (n=16) of body weight. Ibuprofen was dissolved in corn oil. The doses was based on the therapeutic doses of Ibuprofen of 100; 300 and 600 mg used in humans, and the equivalent doses for rats were calculated based on body surface area (Reagan-Shaw et al., 2007). Experiments were conducted in the last week of pregnancy (gestational day (GD) 15-21) until the end of lactation (post-natal day (PND) 1-21) by gavage. The period of the treatment coincided with the critical window of hypothalamic sexual differentiation (MacLusky and Naftolin, 1981). In this experimental design, the pups were exposed to ibuprofen via placenta (gestacional period) and during breastfeeding (after birth). The treatment lasted until the end of the lactation because in this period the brain is rapidly growing, with neuronal migration and differentiation, synaptogenesis, glial multiplication and myelinization, being considered a critical stage of neuronal maturation (Morgane et al., 1993). During the treatment, the body weight, maternal behavior and the food and water consumption was monitored.

After birth, on PND 1, the number of pups per litter was reduced to eight, 4 males and 4 females. The male offspring was weaned on PND22 and maintained until adulthood for evaluation of reproductive parameters. For each set of experiments, one male rat from each litter was sampled in order to prevent variation due to litter effects.

Initial development and external examination at puberty (n=11 – 16)

Rats pups were weighed and anogenital distance measured at PND1, 13 and 22 in all male offspring. AGD was normalized against the cube root of body weight (Gallavan et al,1999). These same rats were examined for testicular descent by scrotal palpation, starting from PND15. From PND35, preputial separation (retraction of the penile gland) was verified daily by manual retraction of the prepuce.

Organ Weights (n=10)

At PND 90, male rats from each group (10/group; 1 male/litter) were weighed and killed by CO_2 inhalation and decapitated for blood collection for hormonal dosage. Organs such as testis, epididymis, seminal vesicle (full), vas deferens, prostate, adrenal, liver and kidney were dissected and weighed on analytical balance. The cauda of the right epididymis were used for analysis of sperm motility.

Sperm motility (n=5)

Analysis of motility from cauda epididymis sperm was performed by computer-assisted sperm analysis (CASA) using CEROS II system equipped with miniTherm stage warmer (Hamilton-Thorne, Bervely, MA, USA). For sperm isolation, cauda epididymis was dissected, rinsed with 0.1 M PBS pH 7.4, and placed in 3 ml of HTF medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 0.75% BSA at 37°C under 5% CO_2 and air. Four to six holes were carefully made in the cauda epididymis using a 30-G needle, allowing sperm release into the medium. After 10 min of incubation, tissue was removed and aliquots of sperm suspension were diluted 1:20 to 1:30 into fresh medium to a concentration of ~2×10^5 sperm/ml. A 15-µl aliquot of

diluted sperm suspension was placed onto 80 μm 2XCELL slides (Hamilton Thorne) for motility assessment. Sperm tracks were captured at 37 °C with a frame rate acquisition of 60 Hz for 1 s (45 frames captured) and 4X negative-phase contrast objective using default instrument settings. We recorded at least 200 spermatozoa and 10 fields for each sample analyzed. We used the video playback to remove mistracked spermatozoa, as may occur due to collisions and false-negative debris detection. We recorded sperm tracks and kinematics parameters individually and removed sperm tracks with less than 22 acquisition points from subsequent analysis. We evaluated the following sperm kinematics parameters as provided by the CASA system: average path velocity (VAP; μm/s), straight line velocity (VSL; μm/s), curvilinear velocity (VCL; μm/s), lateral head displacement (AHL; μm/s), straightness (STR; %), and linearity (LIN; %). Sperm tracks were classified as motile, progressive, slow and static; motile tracks were classified as progressive if STR ≥ 65% and VAP ≥ 100 μm/s, and slow if VAP ≤ 20 μm/s and VSL ≤ 30 μm/s (Silva et al., 2018).

Serum testosterone levels (n= 11-16)

Blood samples (11 – 16 animals/ group) were collected by decapitation and the serum was obtained by centrifugation (2400 rpm for 20 min at 4º C). Serum testosterone levels were determined by radioimmunoassay (RIA), using a commercial solid-phase radioimmunoassay kit (IM 1087) from Beckman Coulter, Inc., using a PerkinElmer's automatic gamma counter. All samples were measured within the same assay to avoid the inter-assay errors. Intra-assay variability was 4%.

Fertility Test - Natural Mating (n= 10-11)

For the evaluation of fertility, another males rats on PND 90 (10 -11 animals/ group) were paired with females, placed in their cages (one female per male), late in the afternoon. On the following morning, vaginal smears were collected and the day that sperm were found in the smear was determined to be GD0. On the GD20 the females were killed by CO_2 inhalation followed by decapitation. After collection of the uterus and ovaries, the numbers of corpora lutea, implants, reabsorptions, 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.

Sexual behavior

Fifteen days after fertility test, the adult rats, now sexually experienced were anesthetized with sodium Ketamine and Xylazine (25 and 10 mg/kg, respectively) and bilaterally castrated. The right testis and epididymis removed were stored for sperm analysis.

Then, all of these males received testosterone cipionate (Sigma Pharma) at 1 mg/day, sc, 3 times a week, for 2 weeks (Ribeiro and Pereira, 2005). The testosterone replacement schedule was set up so that the first injection was given on the day after orchidectomy, and the last one was always applied 24h before the male sexual behavior test. The evaluation of sexual behavior was performed under red-light illumination

during the dark phase of the diurnal cycle. The female sexual behavior was assessed in the same experimental animals 15 days after the male sexual behavior test.

The castration and hormone replacement is used to test whether the animals respond to exogenous testosterone and estradiol, and the presence of male or female sexual behaviour, indicating whether the hypothalamus is masculinised and defeminised. Moreover, this procedure ensures that androgen levels in the rats are similar, excluding the possibility of hormone deficiency, in the absence of male sexual behavior (Piffer et al., 2009).

Male sexual behavior

Male rats were placed individually in cages of polycarbonate crystal, measuring 44 ×31 ×16 cm, 10 min before introduction of 1 adult female in natural estrus (sexually receptive) determined by vaginal smear. The animals were observed in the dark period of the cycle with the aid of red lamps. The following parameters were observed for 40 min: latency to the first mount, intromission, and ejaculation; number of intromissions until the first ejaculation; latency of the first post-ejaculatory intromission; number of postejaculatory intromissions; and number of ejaculations (Ågmo, 1997). The males that did not mount in the initial 10 min were considered sexually inactive (Gerardin et al., 2005; Pereira et al., 2006; Oliva et al., 2006).

Female sexual behavior

Twenty-four hours prior to the test, experimental males were treated with estradiol benzoate (Sigma Co., USA) at 20 μg/kg, ip (Ribeiro and Pereira, 2005) since the female behavior is dependent on estrogen receptors in the brain and their stimulation

by the female hormone. A sexually experienced intact male rat was first placed into an acrylic cage for 10 min for adaptation and then cohabited with each experimental male. Female-typical behaviors (lordosis and mount acceptance) were scored over a period of 10 min.

Sexual partner preference

The assessment of sexual preference was performed on a rectangular arena 50 × 50 × 100 cm (H × W × L) with two small arenas (25 × 15 cm). The small arenas were diagonally opposite to each other and contained stimulus animals: a sexually active male (gonadally intact) was place in one of the small arenas and an oestrus female was placed in the other. The rats were separated by a wire mesh barrier. The floor of the main arena was demarcated in zones in front of each small arena opening, named sexual incentive. Rats were observed for 20 min during the dark phase under red light. The number of visits to each of the sexual incentive zones and the total time spent visiting each zone were quantified (adapted from Vega-Matuszczyk and Larsson, 1995) and a preference score was determined by subtracting the time spent in the zone containing the sexually active male from the time spent in the female zone. A positive score indicates preference for the female, whereas a negative score indicates preference for the male.

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

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) and sperm in the caput/corpus and cauda epididymis were counted as described previously by Robb et al. (1978), with adaptations adopted by Fernandes et al. (2007). Briefly, the testis, decapsulated and weighed soon after collection, was homogenized in 5 ml of

NaCl 0.9% containing Triton X 100 0.5%. After a 10-fold dilution a sample was transferred to Neubauer chambers (4 fields per animal), preceding a count of mature spermatids. To calculate 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 in which these spermatids are present in the seminiferous epithelium. In the same manner, caput/corpus and cauda epididymis portions were cut into small fragments with scissors and homogenized, and sperm counted as described for the testis. The sperm transit time through the epididymis was determined by dividing the number of sperm in each portion by the DSP.

Sperm morphology

For evaluation of sperm morphology, the interior of the left vas deferens of mature rats was washed, with the aid of a syringe and needle, with 1 mL of saline solution, after which histological slides were prepared. Two hundred spermatozoa (heads only or intact sperm) per animal were evaluated for head and/or flagellar defects by phase-contrast microscopy (X 200, total magnification) in wet preparations. 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).

Quantification of SDN-POA volume

Another adults rats (90 days old, n = 5/ group) were deeply anesthetized with an overdose of pentobarbital and perfused transcardially with saline followed by with 4% paraformaldehyde/phosphate buffered saline (PBS), 30ml/min. Brains were post-fixed

in 4% paraformaldehyde/PBS for 24 h, cryoprotected in 20% sucrose/PBS solution, frozen and kept at -40 °C. Brain tissue was sliced in the coronal plane at 40 µm sections on a cryostat (Leica). Tissue was stored in antifreeze solution and maintained at -15 °C. Sections were stained by immunohistochemistry for calbindin D28K, mounting onto gelatin subbed slides and dehydrated through a series of alcohol washes followed by two xylene washes and covers lipped. SDN-POA volumes were analyzed under a Nikon 20× objective using the Neurolucida program package *(version 10, MBF Bioscience, MicroBrightfield, Inc.)*. Tracings were made around the margins of the SDN-POA in both sides of the brain, area quantified by the program, and multiplied by section thickness to obtain volumes.

The left testis and epididymis of these animals were removed for histological analysis.

Histological Evaluation

The left testis and epididymis were immersion fixed in Bouin's fixative (75% picric acid, 25% formaldehyde, and 5% glacial acetic acid) for 24 h and processed for histological analysis. Three nonconsecutive sections (5 µm thick) per animal, separated by 100 µm distance, were obtained, mounted on glass slides, and stained with H&E. The histological evaluation of organs was quantitatively for testis and qualitatively for epididymis. Sertoli cells nuclei were counted in 20 cross-sections of seminiferous tubules per rat, under a light microscope, at 400x magnification (Nassr et al., 2010, with modifications).

Another three sections per animal, were stained with Periodic Acid-Schiff (PAS) to assess spermatogenesis kinetics. Two hundred random tubular sections per animal in the testis cross-sections were classified into four categories: stages I–VI, VII–VIII, IX–

XIII, and XIV of the seminiferous epithelium cycle, according to Leblond and Clemont (1952), under a light microscope at 200x magnification. Adult Leydig cells (ALC) were also evaluated, kariometric analyzes were performed in 50 random circular or elliptical nuclei of ALC per animal (Fichna and Malendowicz, 1975; Mantovani and Fucic, 2014). Major (D) and minor (d) diameter of the cell were obtained using a Nikon Eclipse E200MV, Infinity 1 Camera. Thereafter, the medium diameter (M) was calculated using the formula M=(D + d)/2 and the volume (V) was obtained with the following formula: $V= \pi \times {}^{1}/_{6} \times M^{3}$ (Cury et al., 2006).

Statistical Analysis

Results are expressed as the mean ± SEM. or as median values with the interquartile range given in parentheses. Statistical tests consisted of analysis of variance (ANOVA) with a post hoc Tukey–Kramer test or the Kruskal–Wallis with a post hoc Dunn test. Two-sided $P \leq 0.05$ was considered significant.

Results

Females (pregnant or lactating) exposed to ibuprofen showed no statistical differences in the consumption of water and food, maternal behavior, biochemical parameters or weight gain during treatment compared to the control (supplementary material).

In utero and lactational exposure to ibuprofen resulted in a significant reduction in the body weight at PND 1 that remained until the end of the treatment in the male offspring exposed to a higher dose (60 mg/kg) (Table 1). The same group had a delay in the age of testicular descent (Figure 1A). The animals exposed to the lowest dose (10

mg/kg) had a decrease in relative anogenital distance at PND 13 and 22 (Table 1), and a significant retard in the onset of puberty was determined by preputial separation (Figure 1B).

At PND 90, there were no significant differences among experimental groups in body weight and relative and absolute organs weight (Table 2). The volume of Leydig cells was reduced around 20% in all groups exposed to ibuprofen (Figure 2 A-D, F) the same was observed with testosterone levels, where all groups presented a reduction in relation to control group (10 mg/kg: 29%; 30 mg/kg: 28% and 60 mg/kg: 40%), however this reduction was statistically significant only at the highest dose group (Figure 2E).

The exposure to ibuprofen did not alter the daily sperm production, the number of sperm in the caput/corpus and cauda epididymis, sperm transit time, number of Sertoli cells nuclei or the dynamics of spermatogenesis (Table 3). However, there was a significant difference in the percentage of normal and abnormal spermatozoa (Figure 3A), although the sperm motility was not affected (Figure 3B).

In the fertility test, none of the parameters investigated were markedly different among groups (Table 4). Figure 4A shows that all experimental groups showed male sexual behavior. Although both the lowest and the highest dose group presented female sexual behavior, only the 60 mg/kg group was statistically significant, in which 45% of the animals presented female sexual behavior (Figure 4B), i.e., lordosis and/or accepted rides. Exposure to ibuprofen also compromised the male sexual behavior, because these animals (60 mg/kg group) had decreased latency to the first mount and all the groups treated had decrease in latency time for first intromission after ejaculation (Table 5). Sexual preference was not altered (Figure 5).

The total volume of sexually dimorphic nucleus of the preoptic area of the hypothalamus was reduced in the group exposed to the highest dose, however this difference was not statistically significant (Figure 6).

Discussion

During critical periods of development, organs and systems are susceptible and an adverse prenatal environment permanently 'programs' physiology and increases the risk of disorders in adulthood (Seckl and Holmes, 2007; Barker and Thornburg, 2013). We reported here, for the first time, that in rat experimental model, maternal exposure to ibuprofen during the critical windows of development adversely impact hypothalamus–pituitary–gonadal axis, sexual maturation and reproductive function.

The antiandrogenic effect of ibuprofen observed in our study may have been the main factor involved on negative impacts observed on sexual maturation and in the reproductive function. It is well established that the neuroendocrine pathways that regulate gonadotropin release in rodents is profoundly influenced by neonatal estrogens. These estrogens are aromatized from testicular androgens and are required to organize the male rodent hypothalamic–pituitary–gonadal axis (HPG) as well as other aspects of reproductive physiology and behavior (Corbier et al., 1978, 1992; Weisz and Ward, 1980; MacLusky and Naftolin, 1981; DonCarlos et al., 1995; Simerly, 2002).

The reduction in the birth weight observed in the present study may have contributed to the delay in the age of testicular descent, because lower birth weight is an important marker that may be correlated with fetal programming, once is the first sign that alterations occurred during the intrauterine development (Bertram and Hanson, 2002; Hanson and Gluckman, 2008). Furthermore epidemiological studies reported the

consumption of ibuprofen during pregnancy is associated with an increased risk of cryptorchidism (Kristensen et al., 2011; Snijder et al., 2012).

Our data showed that maternal exposure to ibuprofen affected the programming of HPG axis, since we observed a reduction in testosterone levels in all treated groups. Despite this reduction have been significant only in the higher dose group, the fact of the other groups show a decrease close to 30% certainly not be totally ignored. The decrease around 20% in the volume of Leydig cells after exposure to ibuprofen corroborates the decreased of testosterone concentration because testosterone levels are directly related to the size of Leydig cells (França et al., 2005). Several studies have shown that NSAIDs have antiandrogenic activity, this has been demonstrated in epidemiological studies, in humans and in experimental models both *in vivo* and *in vitro* (Kristensen et al., 2016); direct evidence was provided in ex vivo culture and xenograft systems with human fetal testes, in this model, ibuprofen suppressed testosterone and Leydig hormone INSL3 levels, with concomitant reduction in the expression of steroidogenic enzymes (Ben-Maamar et al., 2017). Another study, using *ex vivo* organotypic models from rat testes, testosterone production was inhibited by paracetamol, acetylsalicylic acid and indomethacin (Kristensen et al., 2011; 2012). Negative effect of NSAID on hormonal regulation was also observed in zebrafish experimental model, where the ibuprofen caused alteration in plasma sex hormone levels as well as in gene transcription in the HPG axis (Ji et al., 2013).

Reduction in the anogenital distance (AGD), observed in this study, reflects an inadequate action or release of testosterone and is an early indication of impaired sexual activity in adulthood (Keshet and Weinstock, 1995; Gerardin et al., 2008). In mammals the AGD is a sexually dimorphic external marker of masculinization (Marois, 1968; Clark et al. 1990; Rhess et al. 1997), besides being a valid biomarker to assess the

effects of an adverse environment reproductive development from fetal to adult life (Thankamony et al., 2016), shorter AGD also is associated with lower fertility, semen quality and testosterone levels (Eisenberg et al., 2011, 2012; Eisenberg and Lipshultz, 2015; Mendiola et al., 2015) and is a predictor of changes in the SDN-POA (Faber and Hughes, 1992). This alteration may have contributed to the delay of puberty onset observed in the same group, since in male, androgens play the more important role in the timing of pubertal onset. Preputial separation is an event necessary for complete copulatory behavior that can be used as an index of mate pubertal development (Korenbrot et al., 1997; Mantovani and Fucic, 2014).

Ibuprofen exposure did not affect the fertility of rats after natural mating, despite the changes in sperm morphology. However, it should be emphasized that changes in sperm parameters are not a direct measure of fertility (Neubert, 1997), since in some rodent species, fertility is compromised only if there is a reduction of approximately 90% in sperm production, which is not the case in men, in whom minor changes could have serious consequences for fertility (Zenick et al. 1994).

Regarding sexual behavior, in the present study, all rats exposed to ibuprofen presented male sexual behavior, but changes in sexual performance were observed, such as a decreased in latency to the first mount and reduction in latency of the first post-ejaculation intromission. These changes are not related to the reduction in testosterone level, since hormone replacement was done before the evaluation of sexual behavior. Testosterone is one of the modulators of male sexual behavior in adult mammals, however, this hormone is not the only factor that regulates the sexual performance. It is necessary normal functioning of the hypothalamic–pituitary–testicular axis for normal sexual behavior (Gerardin et al., 2005). However, after treatment with estradiol benzoate, animals exposed to the highest dose of ibuprofen showed female sexual

behavior, demonstrating an incomplete defeminization, possible due to the presence of functional estrogen receptors in the central nervous system of male rats treated (Pereira, 2003).

In our study, the SDN-POA volume showed slightly reduced in the group exposed to higher dose of ibuprofen, but this reduction was not statistically significant. Changes in the volumes of sexually dimorphic brain nuclei are often used as a biomarker for developmental disruption. However, these morphological analyses not always predict reliably the interruption of cellular phenotype or neuronal function (Patisaul et al., 2007). It is important to emphasize that the volume of a nucleus is not the only existing sexual neuroanatomic difference; sexually dimorphic differences also include the number of cells, connectivity, morphology, physiology, neurotransmitter phenotype and molecular signaling, all of which are determined by the action of steroid hormones (Lenz and McCarthy, 2010). This has been confirmed in another studies, in which treatment did not affect the total volume of the SDN-POA, however, altered the number of immunoreactive cells to the calcium-binding protein, calbindin (protein used to delimit the borders of SDN) (Amateau and McCarthy, 2004; Patisaul et al., 2007). In this sense, new analyzes should be performed to evaluate other parameters among those mentioned above to reinforce our hypothesis.

A non-monotonic dose response (NMDR), as a 'U' shaped curve, was observed in this study. This curve pattern has been frequently reported actually in experimental studies, mainly for natural hormones and endocrine disrupting chemicals (EDCs), in a variety of biological systems including cultured cells, whole organ cultures, laboratory animals and human populations (Vandenberg, 2014). However, that type of relationship is not exclusive to EDCs and is also observed for chemicals substances that do not act on the endocrine system, and can be elicited by non-chemicals stressors, such as

ionizing radiations (Calabrese and Blain, 2005; Kendig et al., 2010). Studies have shown that NMDR relationships can result from a variety of mechanisms, such as opposing effects induced by multiple receptors differing by their affinity, receptor desensitization, negative feedback with increasing dose, or dose-dependent metabolism modulation (Lagarde et al., 2015). In this way, new analyzes will be performed to evaluate which mechanisms may be related to the effects triggered by these doses.

Future studies will further explore the effects neurochemical in the medial preoptic nucleus on the disruption of sexual behavior and the molecular mechanisms underlying adversely effects showed after ibuprofen treatment during brain sexual differentiation.

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Figures and Tables

Table 1. Body weight and relative anogenital distance at postnatal days (PND) 1, 13 and 22 of the male offspring of the control group and groups exposed to 10, 30 and 60 mg/kg of ibuprofen.

Parameters	**Experimental groups**			
	Control	**10 mg/kg**	**30 mg/kg**	**60 mg/kg**
Body weight (g)				
PND 1	7.08 ± 0.07	7.13 ± 0.07	7.04 ± 0.07	6.80 ± 0.08*
PND 13	29.78 ± 0.40	29.53 ± 0.30	30.22 ± 0.34	28.20±0.30**
PND 22	56.54 ± 0.82	54.94 ± 0.70	56.51 ± 0.73	54.16 ± 0.60*
Relative anogenital distance (mm/g)				
PND 1	2.39 ± 0.02	2.36 ± 0.02	2.40 ± 0.02	2.41 ± 0.02
PND 13	3.52 ± 0.03	3.36 ± 0.04**	3.43 ± 0.03	3.48 ± 0.03
PND 22	5.22 ± 0.04	5.04 ± 0.05*	5.11 ± 0.05	5.21 ± 0.05

Values expressed as mean ± SEM of 11 - 16 litters per group. ANOVA with "a posteriori" of Tukey. *p<0.05 and **p <0.01 compared to the control group.

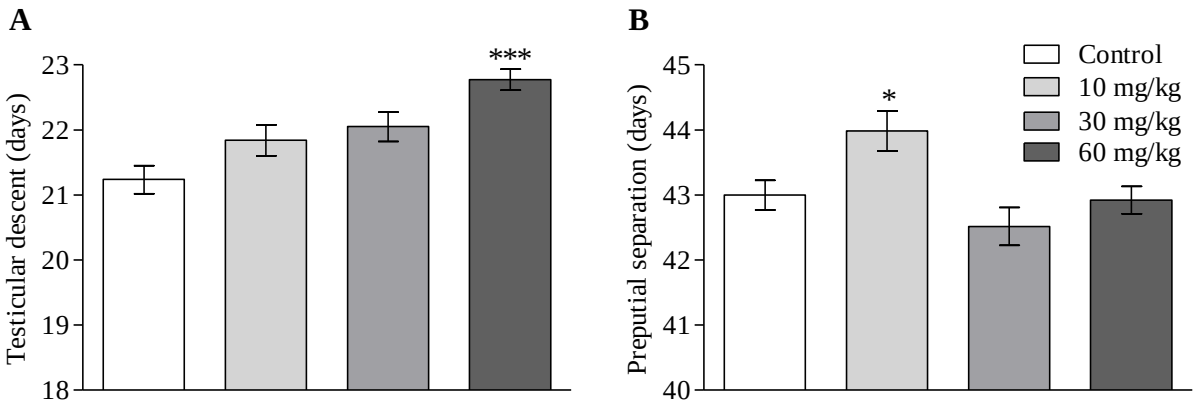

Figure 1. Age at the time of testicular descent (A) and preputial separation (B) of male offspring of the control group and groups exposed to 10, 30, 60 mg/kg of ibuprofen. Values expressed as mean ± SEM of 11 - 16 litters per group. ANOVA with "a posteriori" of Tukey. *p<0.05 and ***p <0.001 compared to the control group.

Table 2. Final body weight, absolute and relative weight of organs of adult male rats of the control group and groups exposed to 10, 30 and 60 mg/kg of ibuprofen.

Parameters	Experimental groups			
	Control	**10 mg/kg**	**30 mg/kg**	**60 mg/kg**
Body weight (g)	510.40 ± 44.93	540.18 ± 45.73	528.67 ± 56.18	525.04 ± 58.10
Liver (g)	18.66 ± 3.21	18.78 ± 3.81	17.85 ± 2.15	19.12 ± 3.67
Liver (g/100g)	3.65 ± 0.45	3.47 ± 0.60	3.34 ± 0.33	3.63 ± 0.47
Kidney (g)	1.88 ± 0.22	2.04 ± 0.38	1.81 ± 0.30	1.91 ± 0.23
Kidney (g/100g)	0.37 ± 0.04	0.38 ± 0.07	0.34 ± 0.05	0.36 ± 0.03
Adrenal gland (mg)	36.40 ± 2.32	33.10 ± 2.40	34.22 ± 2.57	31.70 ± 2.49
Adrenal gland (mg/100g)	7.10 ± 0.46	6.10 ± 0.43	6.22 ± 0.28	6.10 ± 0.48
Spleen (g)	0.73 ± 0.02	0.81 ± 0.05	0.75 ± 0.05	0.78 ± 0.03
Spleen (g/100g)	0.14 ± 0.00	0.15 ± 0.01	0.14 ± 0.01	0.15 ± 0.01
Heart (g)	1.90 ± 0.18	1.84 ± 0.11	1.72 ± 0.06	1.77 ± 0.06
Heart (g/100g)	0.37 ± 0.03	0.34 ± 0.02	0.33 ± 0.01	0.34 ± 0.01
Lung (g)	2.73 ± 0.31	2.29 ± 0.20	2.44 ± 0.17	2.50 ± 0.20
Lung (g/100g)	0.54 ± 0.06	0.43 ± 0.04	0.47 ± 0.04	0.48 ± 0.05
Testis (g)	1.85 ± 0.11	1.83 ± 0.25	1.85 ± 0.13	1.64 ± 0.47
Testis (g/100g)	0.36 ± 0.02	0.34 ± 0.04	0.35 ± 0.04	0.31 ± 0.09
Epididymis (g)	0.73 ± 0.06	0.70 ± 0.11	0.73 ± 0.09	0.69 ± 0.14
Epididymis (g/100g)	0.14 ± 0.02	0.13 ± 0.02	0.14 ± 0.01	0.13 ± 0.02
Vans deferens (g)	0.12 ± 0.01	0.11 ± 0.02	0.12 ± 0.01	0.12 ± 0.03
Vans deferens (mg/100g)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Seminal gland (g)	1.47 ± 0.23	1.60 ± 0.34	1.48 ± 0.33	1.46 ± 0.35
Seminal gland (g/100g)	0.29 ± 0.03	0.30 ± 0.07	0.29 ± 0.06	0.27 ± 0.04
Prostate (g)	0.74 ± 0.06	0.78 ± 0.11	0.74 ± 0.05	0.67 ± 0.05
Prostate (g/100g)	0.14 ± 0.01	0.14 ± 0.02	0.14 ± 0.01	0.13 ± 0.01

Values expressed as mean ± SEM of 10 animals per group. ANOVA with "a posteriori" of Tukey.

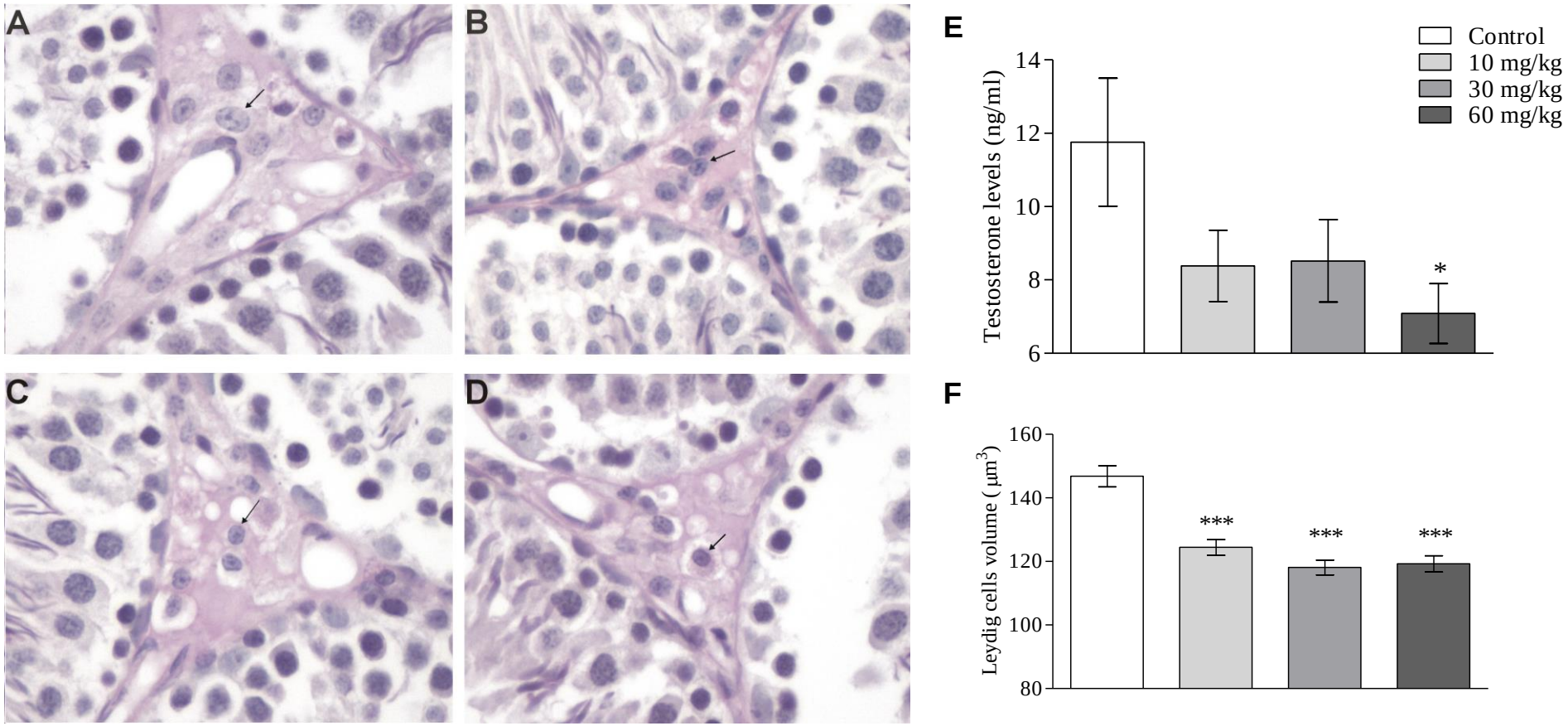

Figure 2. (E) Testosterone serum levels (n= 11-16 animals/group) and (F) Leydig cells nucleus volume (n= 7 animals/group) of adult male offspring of control group and groups exposed ibuprofen. (A–D) Transversal histological sections from adult testis; A: Control group; B: 10 mg/kg; C: 30 mg/kg; D: 60 mg/kg. Animals exposed to ibuprofen had a decrease in the volume of Leydig cell nuclei (arrows). Periodic acid-Schiff, 40x. Values expressed as mean ± SEM. ANOVA with "a posteriori" of Tukey. *p<0.05 ***p<0.001 compared to control group.

Table 3. Dynamics of spermatogenesis, Sertoli cells nuclei number and sperm counts of adult male rats of the control group and groups exposed to 10, 30, 60 mg/kg of ibuprofen.

Parameters	Experimental Groups			
	Control	**10 mg/kg**	**30 mg/kg**	**60 mg/kg**
# Dynamics of spermatogenesis				
I – VI (%)	46.00 (42.50 – 61.50)	43.00 (42.00 – 57.00)	46.00 (40.50 – 56.00)	45.00 (43.00 – 52.50)
VII – VIII (%)	22.50 (16.50 – 23.50)	21.00 (20.50 – 29.50)	21.50 (14.50 – 24.00)	25.00 (16.00 – 29.50)
IX – XIII (%)	28.00 (21.00 – 35.50)	27.50 (23.00 – 31.50)	28.00 (26.00 – 39.00)	26.00 (20.00 – 35.00)
XIV (%)	1.00 (0.00 – 5.50)	0.50 (0.00 – 9.00)	3.00 (1.50 – 3.50)	2.50 (1.50 – 4.50)
Sertoli cell nuclei number (n)	15.56 ± 1.12	16.37 ± 1.40	17.31 ± 1.06	15.53 ± 1.10
Sperm count in the testis				
Mature spermatids number ($x10^6$/testis)	181.46 ± 14.28	177.32 ± 16.19	174.14 ± 7.68	179.71 ± 11.13
Relative mature spermatids number ($x10^6$/g/testis)	120.60 ± 6.95	115.84 ± 10.42	120.02 ± 4.51	128.17 ± 7.98
Daily sperm production ($x10^6$/testis/day)	29.75 ± 2.34	29.07 ± 2.65	28.55 ± 1.26	29.46 ± 1.82
Relative daily sperm production ($x10^6$/testis/g/day)	19.77 ± 1.14	18.99 ± 1.71	19.67 ± 0.74	21.01 ± 1.31
Sperm count in epididymis (caput/corpus)				
Sperm number in caput/corpus ($x10^6$/organ)	98.60 ± 6.71	90.87 ± 7.00	95.77 ± 9.19	93.08 ± 9.01
Relative sperm number in caput/corpus ($x10^6$/g/organ)	342.08 ± 29.43	274.50 ± 15.54	265.97 ± 21.33	306.70 ± 27.76
Sperm transit time (days)	3.21 ± 0.32	3.34 ± 0.37	3.35 ± 0.38	3.25 ± 0.33
Sperm count in epididymis (cauda)				
Sperm number in cauda ($x10^6$/organ)	205.17 ± 10.56	188.19 ± 17.33	180.73 ± 9.84	186.75 ± 9.28
Relative sperm number in cauda ($x10^6$/g/organ)	866.39 ± 79.44	726.25 ± 47.98	713.05 ± 40.92	742.50 ± 25.64
Sperm transit time (days)	6.75 ± 0.53	6.83 ± 0.72	6.30 ± 0.37	6.48 ± 0.34

Values expressed as mean ± SEM of 10-11 animals per group. ANOVA with "a posteriori" of Tukey.

#Values expressed as median - interquartile range (Q1-Q3). Kruskal-Wallis with Dunn's posterior test.

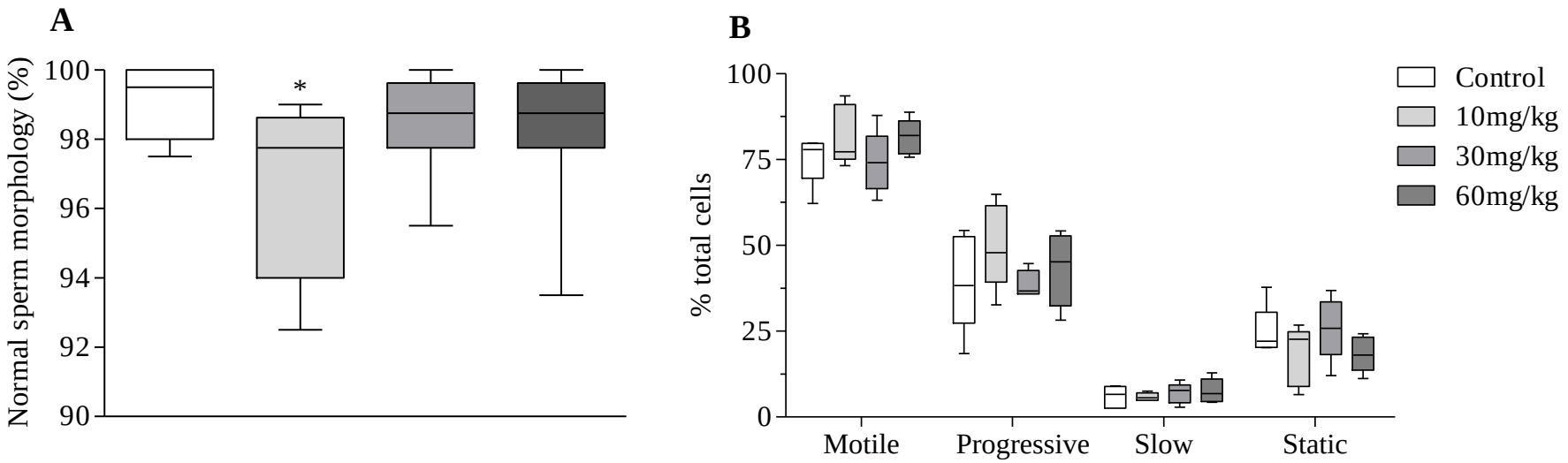

Figure 3. (A) Sperm morphology (n= 10-11 animals/group) and (B) Sperm Motility (n= 5 animals/group) of cauda epididymis sperm of adult male rats of the control group and groups exposed to ibuprofen. Values expressed as median - interquartile range (Q1-Q3). Kruskal-Wallis with Dunn's posterior test. *$p<0.05$ compared to control group.

Table 4. Fertility test after natural mating of adult male rats of the control group and groups exposed to 10, 30, 60 mg/kg of ibuprofen.

Parameters	Experimental groups			
	Control	**10 mg/kg**	**30 mg/kg**	**60 mg/kg**
Pregnancy rate (%)	100	100	100	90.9
# Fertility potential (%)	100 (90-100)	92.31 (58.33-100)	100 (85.71-100)	96.66 (76.92-100)
Final body weight (g)	381.59 ± 6.41	352.31 ± 8.60	369.01 ± 8.26	362.89 ± 9.16
Uterine + fetal weight (g)	63.41 ± 4.27	61.14 ± 4.72	72.70 ± 1.44	59.79 ± 3.33
Fetal weight (g)	3.98 ± 0.07	3.90 ± 0.08	4.10 ± 0.08	3.87 ± 0.03
Placenta weight (g)	0.58 ± 0.02	0.54 ± 0.01	0.55 ± 0.01	0.58 ± 0.03
Number of live fetuses	10.08 ± 0.61	10.54 ± 0.93	12.20 ± 0.29	10.30 ± 0.65
Number of implantations	12.11 ± 0.31	11.27 ± 0.76	12.70 ± 0.33	11.60 ± 0.56
Number of corpora lutea	12.10 ± 0.35	12.70 ± 0.47	13.30 ± 0.37	12.40 ± 0.43
Number of resorptions	1.00 ± 0.28	0.73 ± 0.33	0.50 ± 0.17	1.30 ± 0.54
# Preimplantation loss (%)	0 (0 – 10)	7.41 (0 – 9.09)	0 (0 – 14.29)	3.33 (0 – 22.08)
# Posimplantation loss (%)	7.73 (0 – 27.27)	0 (0 – 28.57)	3.57 (0 – 8.33)	3.33 (0 – 23.08)
Sex ratio (M:F)	1.27 ± 0.19	1.26 ± 0.15	1.65 ± 0.29	0.94 ± 0.18

Values expressed as mean ± SEM of 10-11 animals per group. ANOVA with "a posteriori" of Tukey.

Values expressed as median - interquartile range (Q1-Q3). Kruskal-Wallis with Dunn's posterior test.

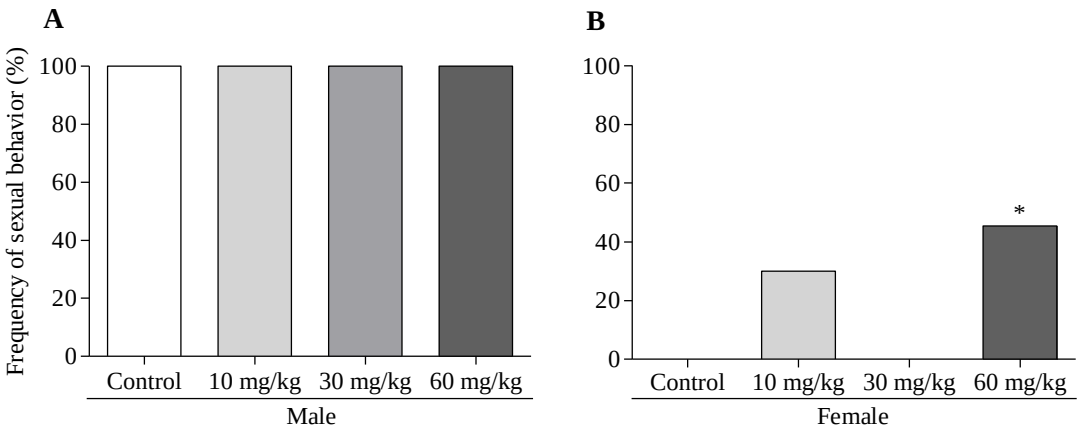

Figure 4. Frequency of control and ibuprofen-treated adult males that showed male sexual behavior after receiving testosterone propionate and that showed female sexual behavior after receiving estradiol benzoate (n =10-11 animals/ group). Kruskal-Wallis with "a posteriori" Dunn. *p<0.05 compared to control group.

Table 5. Male sexual behavior of adult male rats of the control group and groups exposed to 10, 30, 60 mg/kg of ibuprofen.

Parameters	Experimental groups			
	Control	**10 mg/kg**	**30 mg/kg**	**60 mg/kg**
Latency to the first mount (s)	27.75 ± 1.64	18.80 ± 1.80	20.50 ± 3.27	17.30 ± 2.28*
Number of mounts	5.44 ± 1.04	7.20 ± 1.79	4.90 ± 0.64	8.73 ± 1.92
Latency of the first intromission (s)	36.78 ± 6.20	27.40 ± 3.44	28.22 ± 5.59	31.50 ± 10.22
Number of intromissions	17.00 ± 1.41	16.30 ± 1.79	14.40 ± 2.32	19.45 ± 3.27
Latency to the first ejaculation (s)	392.50 ± 58.23	287.78 ± 31.62	310.80 ± 54.62	488.00 ± 82.21
Latency of the first post-ejaculatory mount (s)	363.55 ± 53.96	252.67 ± 28.37	283.11 ± 39.50	252.60 ± 10.01
Number of post-ejaculatory mounts	3.33 ± 0.50	2.10 ± 0.57	3.60 ± 0.67	3.82 ± 0.93
Latency of the first post-ejaculatory intromission (s)	385.00 ± 47.80	261.78 ± 20.90*	247.75 ± 14.29*	262.27 ± 9.08*
Number of post-ejaculatory intromissions	12.11 ± 1.75	10.80 ± 1.24	9.70 ± 1.51	8.60 ± 0.67
Total number of ejaculations	3.33 ± 0.23	3.60 ± 0.27	3.80 ± 0.33	3.45 ± 0.25

Values expressed as mean ± SEM of 10-11 animals per group. ANOVA with "a posteriori" of Tukey. *$p<0.05$ compared to control group.

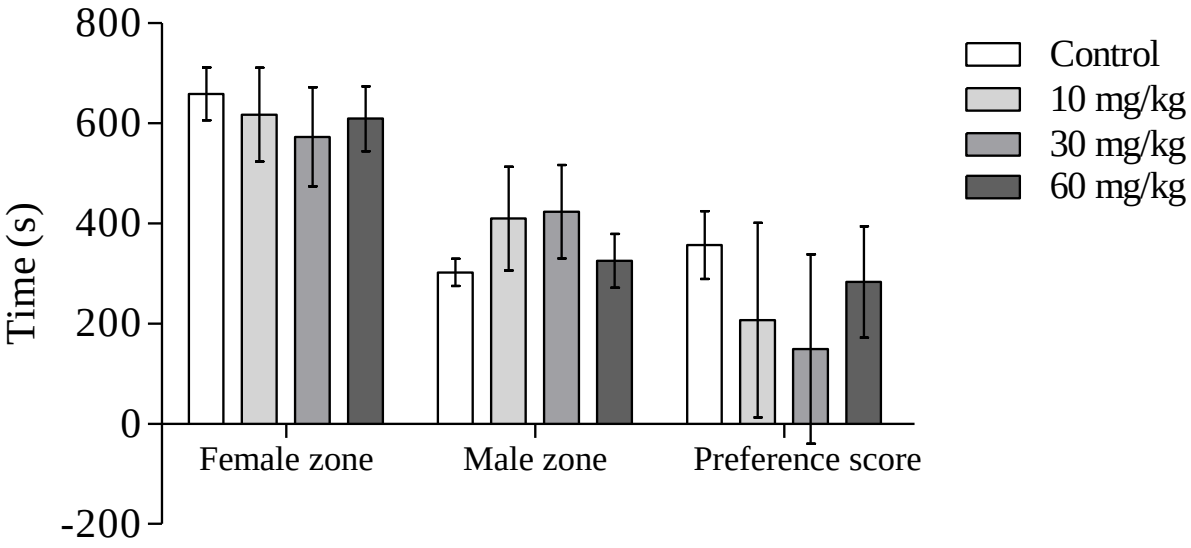

Figure 5. Sexual preference score of adult male rats of the control group and groups exposed to 10, 30, 60 mg/kg of ibuprofen. Values expressed as mean ± SEM of 10-11 animals/ group. ANOVA with "a posteriori" of Tukey.

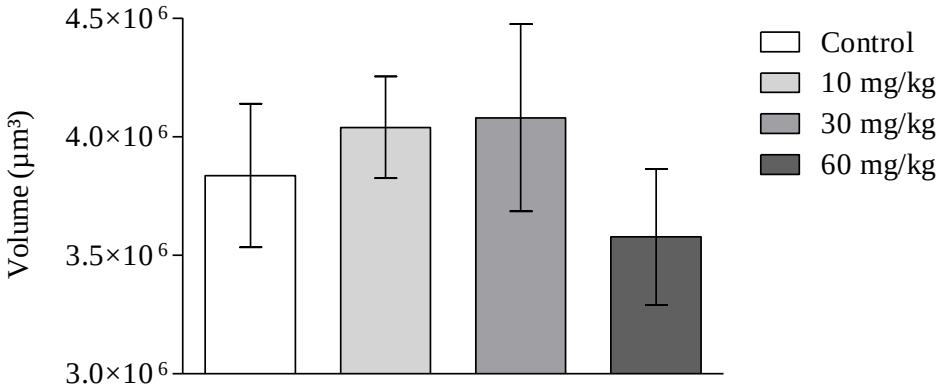

Figure 6. Volume of the sexually dimorphic nucleus of the preoptic area in adult male rats. Values expressed as mean ± SEM of 5 animals per group. ANOVA with "a posteriori" of Tukey.

Supplementary material

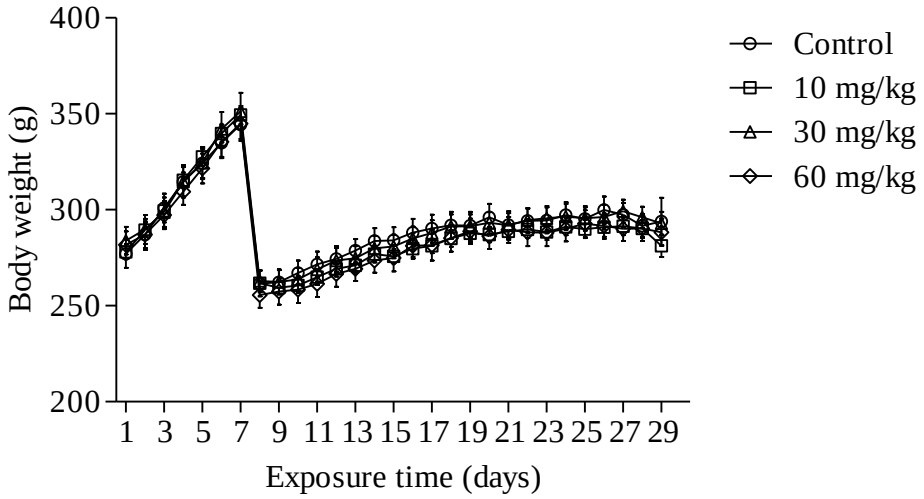

Figure 1. Evolution of body weight (g) to the dams during the treatment. Values expressed as mean ± SEM of 11-16 animals per group. ANOVA with "a posteriori" of Tukey.

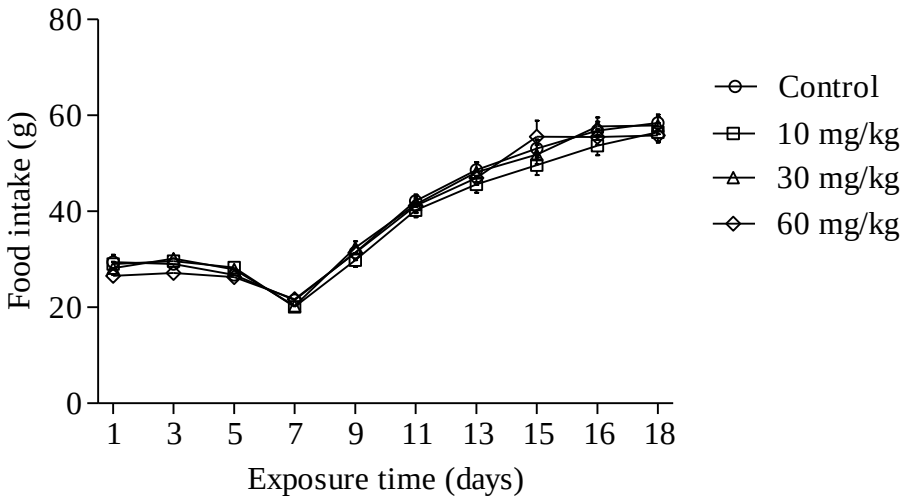

Figure 2. Evolution of food intake (g) to the dams during the treatment. Values expressed as mean ± SEM of 11-16 animals per group. ANOVA with "a posteriori" of Tukey.

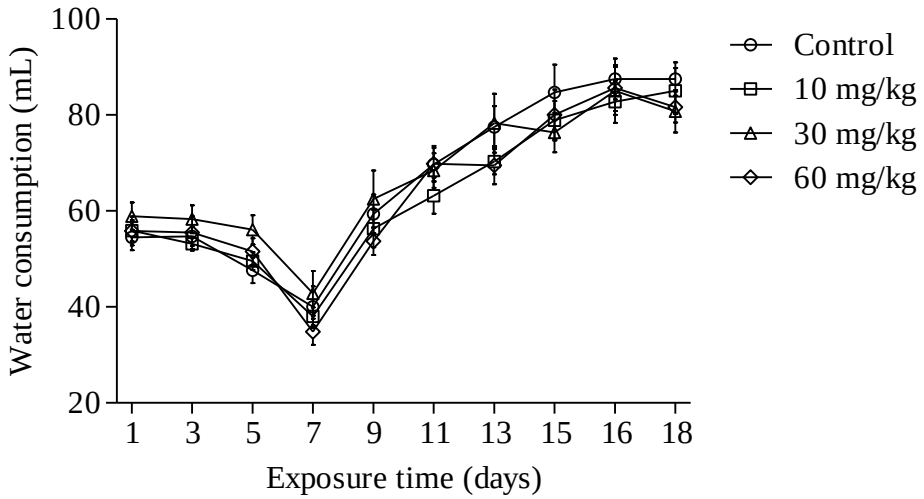

Figure 3. Evolution of water consumption (ml) to the dams during the treatment. Values expressed as mean ± SEM of 11-16 animals per group. ANOVA with "a posteriori" of Tukey.

Table 1 - Biochemical parameters of the dams of the control group and exposed to 10, 30 and 60 mg/kg ibuprofen.

Parameters	Experimental groups			
	Control	**10 mg/kg**	**30 mg/kg**	**60 mg/kg**
AST (U/L)	229.90 ± 25.61	199.77 ± 8.40	193.25 ± 11.41	184.52 ± 7.77
ALT (U/L)	116.74 ± 10.18	91.95 ± 11.32	97.55 ± 10.50	91.97 ± 10.99
Alkaline phosphatase (U/L)	616.16 ± 70.55	587.30 ± 49.64	581.60± 09.32	574.11 ± 36.88
Gamma glutamyltransferase (U/L)	3.57 ± 1.82	5.27 ± 1.35	4.31 ± 2.28	7.11 ± 2.48
Total protein (g/dL)	5.76 ± 0.15	5.82 ± 0.12	5.90 ± 0.12	5.78 ± 0.18
Albumin (g/dL)	4.03 ± 0.11	4.07 ± 0.10	4.12 ± 0.19	3.93 ± 0.15
Globulin (g/dL)	1.74 ± 0.08	1.74 ± 0.08	1.77 ± 0.10	1.87 ± 0.08
Alb/glob ratio	2.36 ± 0.13	2.35 ± 0.11	2.40 ± 0.22	2.13 ± 0.14
Blood urea nitrogen (mg/dL)	58.51 ± 3.26	63.93 ± 4.89	63.16 ± 3.09	67.97 ± 1.64
Creatinine (mg/dL)	0.23 ± 0.01	0.28 ± 0.04	0.22 ± 0.01	0.24 ± 0.01
Sodium (mmol/dL)	132.76 ± 2.06	133.47 ± 1.94	134.75 ± 1.64	134.07 ± 1.27
Potassium (mmol/L)	7.33 ± 0.45	7.47 ± 0.52	7.49 ± 0.16	7.44 ± 0.19
Calcium (mg/dL)	9.63 ± 0.24	10.00 ± 0.61	9.95 ± 0.16	10.02 ± 0.17
Cholesterol (mg/dL)	80.35 ± 3.94	73.47 ± 5.47	78.07 ± 2.55	75.02 ± 3.81
HDL Cholesterol (mg/dL)	59.87 ± 3.80	53.75 ± 5.07	50.26 ± 3.80	47.63 ± 4.31
Glucose (mg/dL)	107.63 ± 2.44	107.17 ± 3.98	115.12 ± 4.13	109.24 ± 6.38

Values expressed as mean ± SEM of 11-16 animals per group. ANOVA with "a posteriori" of Tukey.

Table 2 - Final body weight and absolute and relative organs weights of the dams of the control group and exposed to 10, 30 and 60 mg/kg ibuprofen.

Parameters	**Experimental groups**			
	Control	**10 mg/kg**	**30 mg/kg**	**60 mg/kg**
Body weight (g)	303.53 ± 9.28	280.43 ± 5.11	289.86 ± 8.61	281.67 ± 8.26
Liver (g)	14.12 ± 0.99	13.57 ± 0.63	14.35 ± 0.60	14.40 ± 0.67
Liver (g/100g)	4.81 ± 0.18	4.83 ± 0.19	4.95 ± 0.14	5.10 ± 0.17
Kidney (g)	1.07 ± 0.03	1.08 ± 0.03	1.09 ± 0.03	1.06 ± 0.03
Kidney (g/100g)	0.37 ± 0.01	0.38 ± 0.01	0.38 ± 0.00	0.38 ± 0.01
Adrenal gland (mg)	42.28 ± 2.58	40.00 ± 2.09	47.75 ± 2.62	44.36 ± 2.14
Adrenal gland (mg/100g)	14.55 ± 0.78	14.27 ± 0.72	16.40 ± 0.58	15.72 ± 0.57
Spleen (g)	0.56 ± 0.03	0.58 ± 0.04	0.55 ± 0.02	0.58 ± 0.02
Spleen (g/100g)	0.19 ± 0.00	0.21 ± 0.01	0.19 ± 0.01	0.21 ± 0.01
Heart (g)	1.05 ± 0.06	1.01 ± 0.04	1.00 ± 0.04	0.99 ± 0.04
Heart (g/100g)	0.36 ± 0.01	0.36 ± 0.02	0.34 ± 0.01	0.36 ± 0.02
Lung (g)	1.71 ± 0.19	1.58 ± 0.08	1.51 ± 0.07	1.45 ± 0.10
Lung (g/100g)	0.59 ± 0.07	0.56 ± 0.03	0.52 ± 0.03	0.51 ± 0.02
Ovaries (mg)	81.14 ± 5.47	88.78 ± 6.98	94.75 ± 6.11	81.27 ± 6.70
Ovaries (mg/100g)	27.78 ± 1.29	31.48 ± 2.07	32.69 ± 1.96	28.62 ± 1.98
Uterus+fluid (g)	0.30 ± 0.04	0.29 ± 0.05	0.25 ± 0.01	0.26 ± 0.04
Uterus+fluid (g/100g)	0.10 ± 0.01	0.10 ± 0.02	0.09 ± 0.01	0.09 ± 0.01

Values expressed as mean ± SEM of 11-16 animals per group. ANOVA with "a posteriori" of Tukey.

Table 3. Kinematics parameters of spermatozoa from the cauda epididymis from the control group and exposed to ibuprofen.

Experimental Groups	Sperm tracks	Sperm kinematics parameters					
		VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm/s)	STR (%)	LIN (%)
Control	1532	132.50 ± 5.10	84.60 ± 7.04	325.68 ± 17.43	24.50 ± 0.60	64.04 ± 3.58	26.30 ± 1.46
10 mg/kg	1843	129.74 ± 3.29	89.62 ± 3.78	318.32 ± 12.82	24.24 ± 0.48	68.94 ± 1.51	28.38 ± 0.29
30 mg/kg	1746	126.10 ± 4.85	81.54 ± 2.73	304.14 ± 17.96	23.90 ± 0.62	64.62 ± 1.85	26.80 ± 0.81
60 mg/kg	1947	121.02 ± 8.32	81.40 ± 6.96	300.94 ± 29.61	22.78 ± 0.81	66.58 ± 1.57	27.14 ± 0.45

Values expressed as mean ± SEM of 5 animals per group. ANOVA with "a posteriori" of Tukey. The total number of sperm tracks analyzed is indicated.

VAP= average path velocity; VSL= straight line velocity; VCL = curvilinear velocity; ALH = amplitude of lateral head displacement; STR = straightness; LIN = linearity.

Conclusão

Nossos resultados demonstraram, neste modelo experimental, que a exposição *in utero* e lactacional ao anti-inflamatório ibuprofeno foi capaz de desencadear efeitos adversos sobre o desenvolvimento sexual inicial, parâmetros reprodutivos e comportamentais da prole masculina, indicando que o ibuprofeno pode afetar a programação fetal se for utilizado durante o período crítico de diferenciação sexual hipotalâmica.

Apêndice

As figuras pertencentes a esta seção não foram incluídas no manuscrito, entretanto as análises foram realizadas durante a execução do projeto de pesquisa.

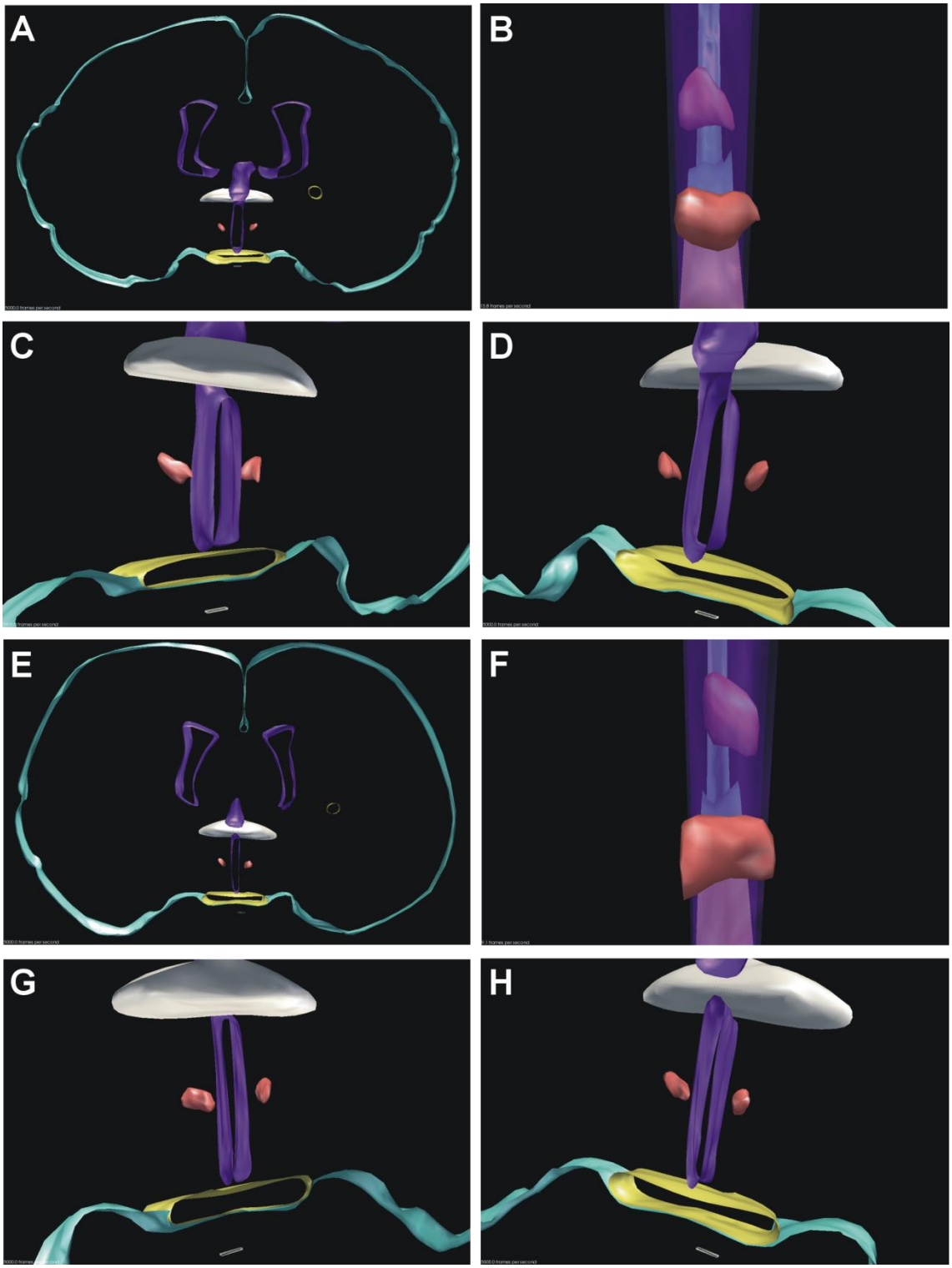

Figura 1- Modelos tridimensionais obtidos através do programa *Neurolucida (versão 10, MBF Bioscience, MicroBrightfield, Inc.)* de animais com 90 dias. Em A, B, C e D imagens de um animal do grupo controle. Em E, F, G e H, imagens de um animal do grupo exposto a dose de 10 mg/kg de ibupofreno. Em A e E, visualização caudal do encéfalo em secção coronal. Em B e F, vista lateral do SDN-POA. Em C e G, vista rostrolateral. Em D e H, vista caudolateral. Contorno externo do encéfalo (azul-claro), ventrículos laterais e 3º ventrículo (roxo), comissura anterior (branco), quiasma óptico (amarelo) e SDN-POA (vermelho).

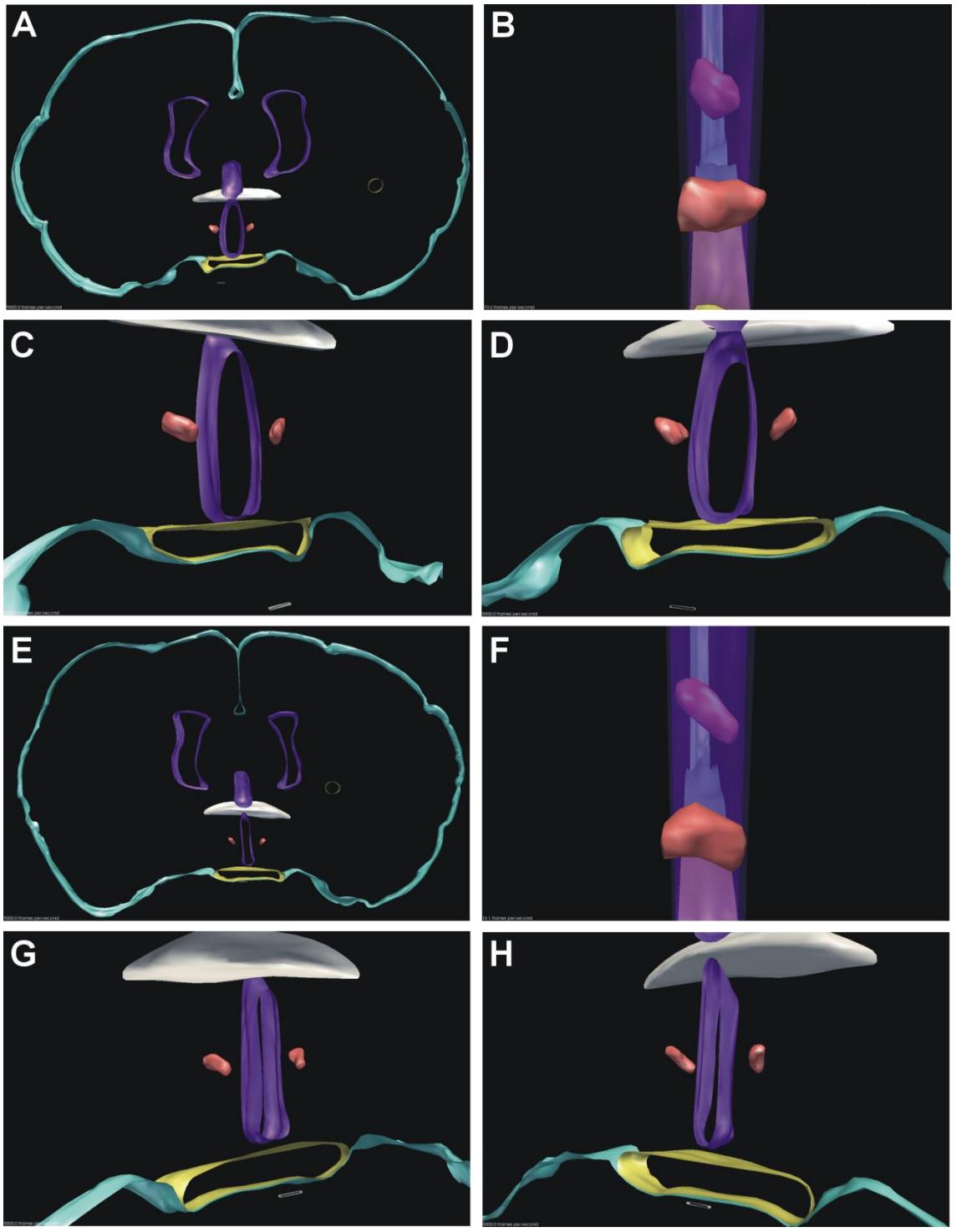

Figura 2- Modelos tridimensionais obtidos através do programa *Neurolucida (versão 10, MBF Bioscience, MicroBrightfield, Inc.)* de animais com 90 dias. Em A, B, C e D imagens de um animal do grupo exposto a dose de 30 mg/kg de ibupofreno. Em E, F, G e H, imagens de um animal do grupo exposto a dose de 60 mg/kg de ibupofreno. Em A e E, visualização caudal do encéfalo em secção coronal. Em B e F, vista lateral do SDN-POA. Em C e G, vista rostrolateral. Em D e H, vista caudolateral. Contorno externo do encéfalo (azul-claro), ventrículos laterais e 3º ventrículo (roxo), comissura anterior (branco), quiasma óptico (amarelo) e SDN-POA (vermelho).

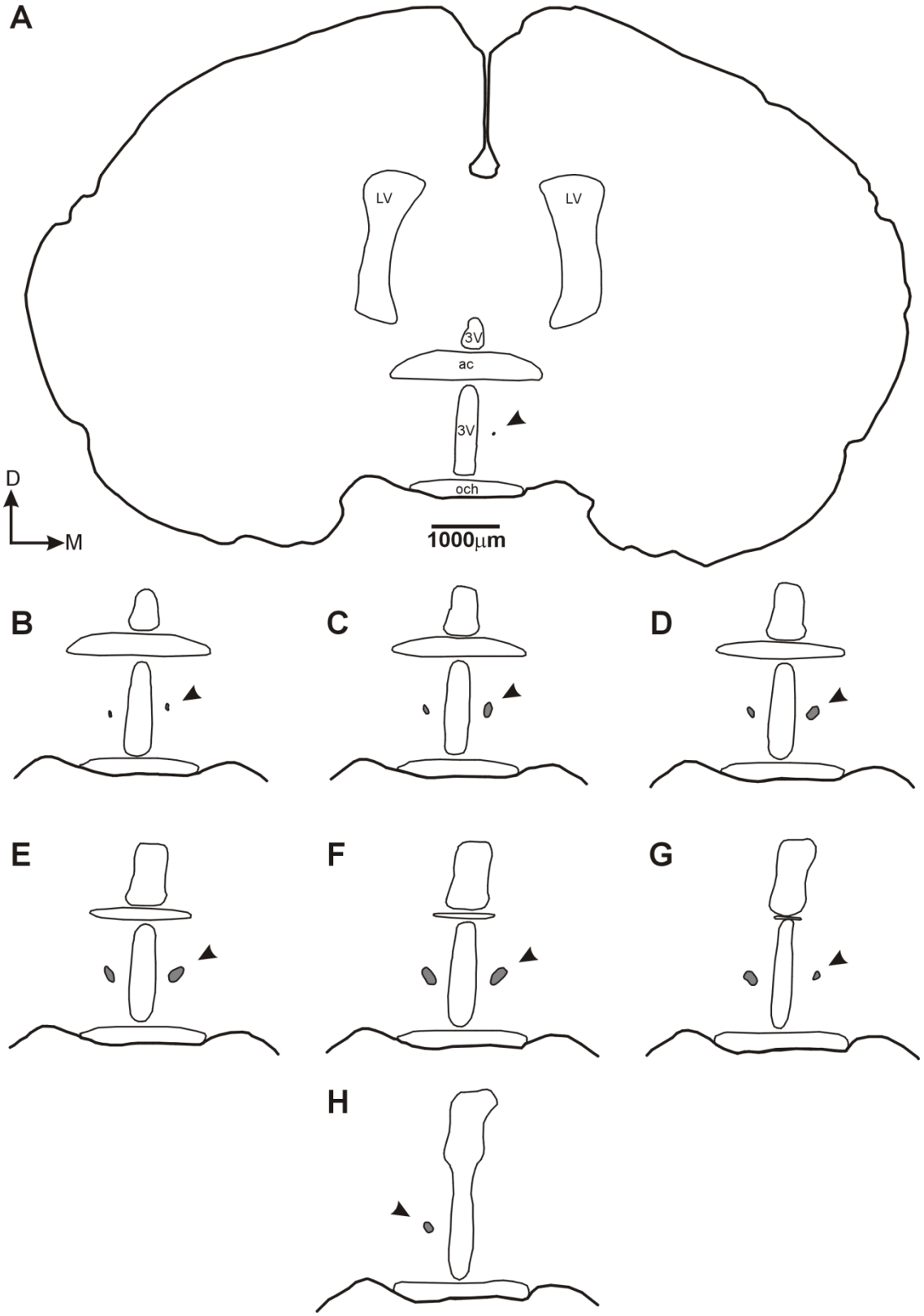

Figura 3- Esquemas representando cortes coronais encéfalo em caso experimental do grupo controle com 90 dias. Em A corte total. Em B, C, D, E, F, G, H porção do hipotálamo. Contornos confeccionados e exportados a partir programa CorelDRAW® graphics suite X3 para serem utilizados na reconstrução tridimensional. A barra de calibração em A é válida para todas as imagens. VL: ventrículo lateral, 3V: 3º ventrículo, ac: comissura anterior, och: quiasma óptico. As setas apontam para o SDN-POA (válido também para as Figuras **4**, **5**, **6**).

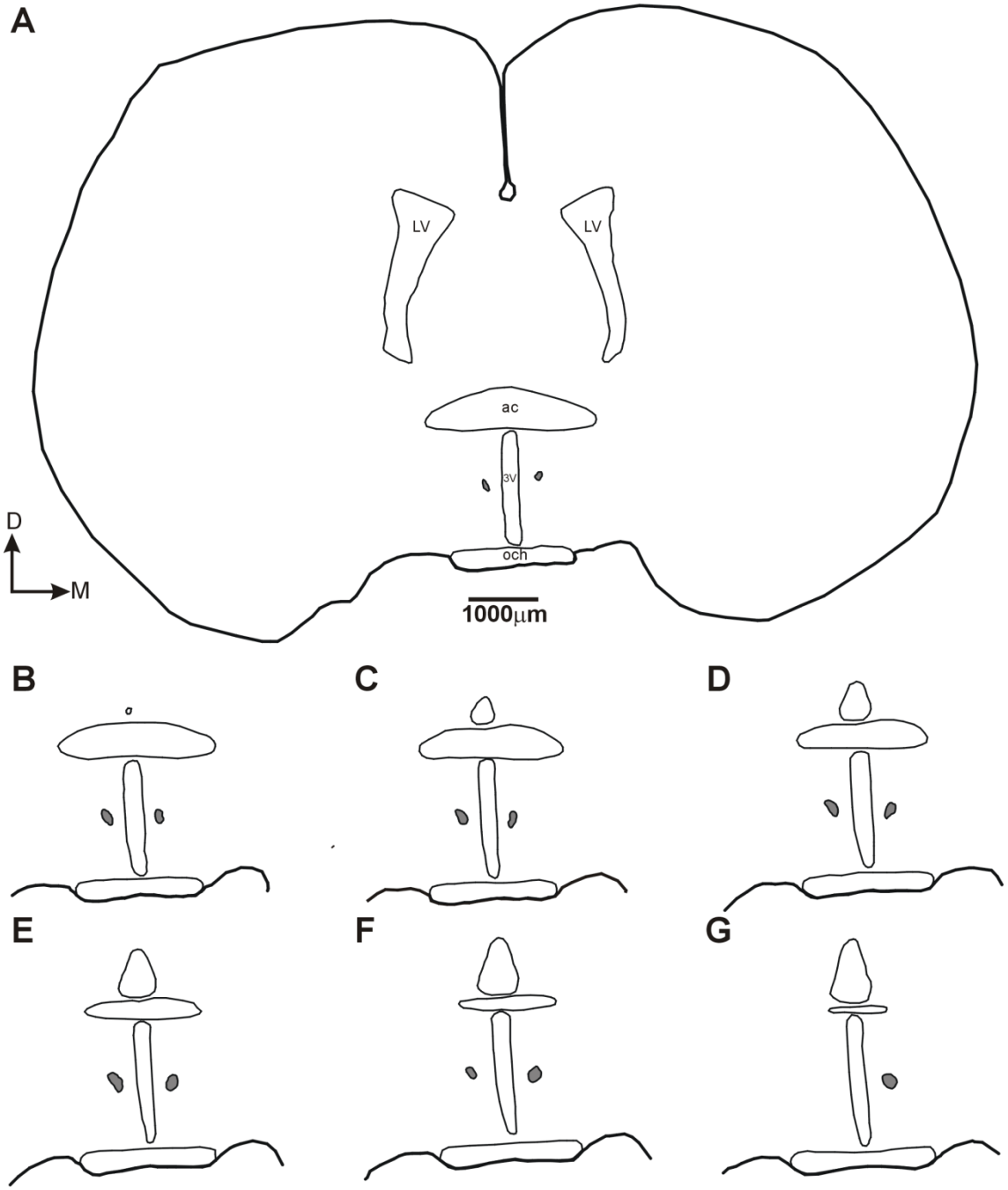

Figura 4- Esquemas representando cortes coronais encéfalo em caso experimental com 90 dias do grupo exposto a dose de 10 mg/kg de ibuprofeno. Em A corte total. Em B, C, D, E, F, G porção do hipotálamo. Contornos confeccionados e exportados a partir programa CorelDRAW® graphics suite X3 para serem utilizados na reconstrução tridimensional. A barra de calibração em A é válida para todas as imagens. VL: ventrículo lateral, 3V: 3º ventrículo, ac: comissura anterior, och: quiasma óptico.

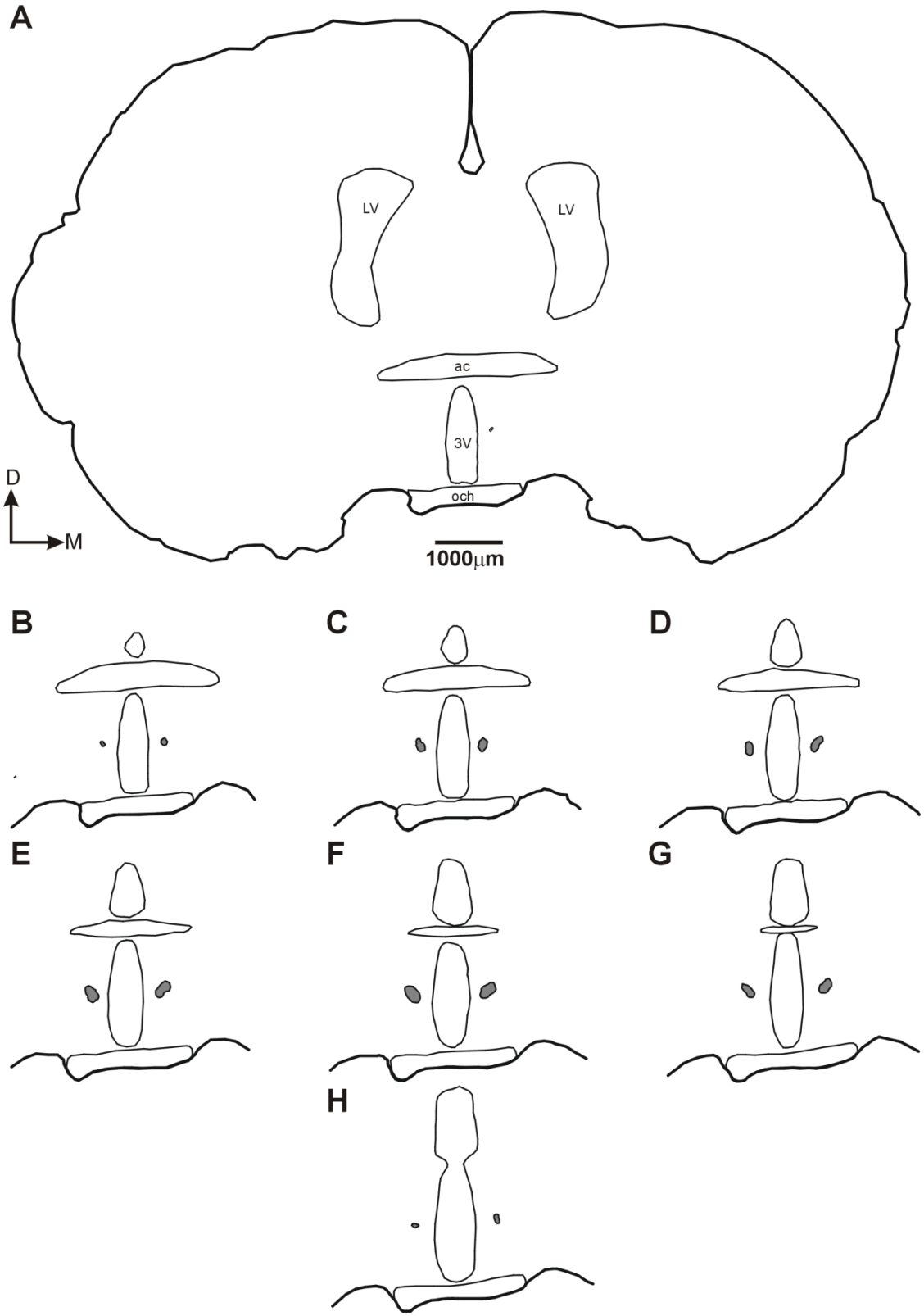

Figura 5- Esquemas representando cortes coronais encéfalo em caso experimental com 90 dias do grupo exposto a dose de 30 mg/kg de ibuprofeno. Em A corte total. Em B, C, D, E, F, G porção do hipotálamo. Contornos confeccionados e exportados a partir programa CorelDRAW® graphics suite X3 para serem utilizados na reconstrução tridimensional. A barra de calibração em A é válida para todas as imagens. VL: ventrículo lateral, 3V: 3º ventrículo, ac: comissura anterior, och: quiasma óptico.

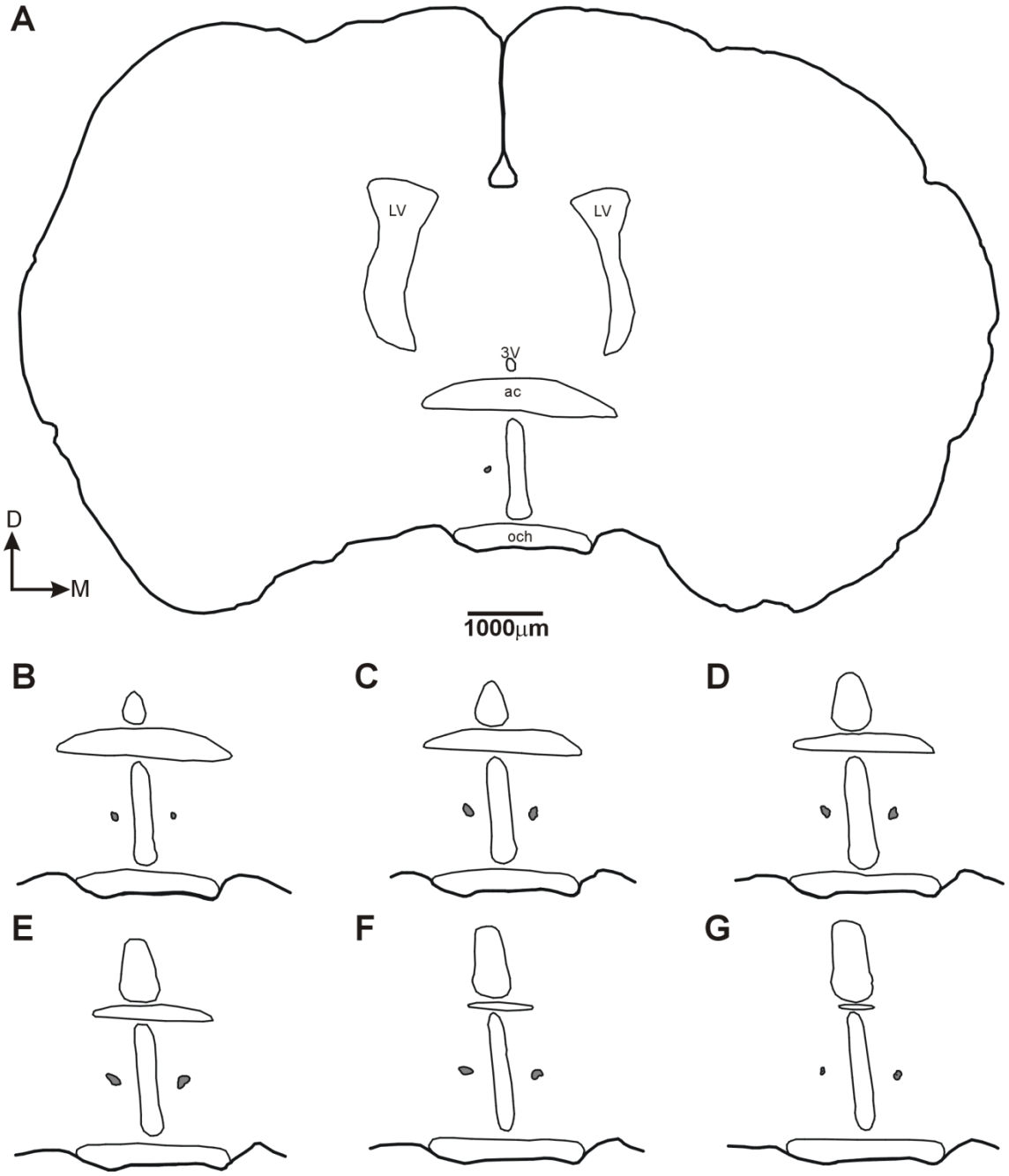

Figura 6- Esquemas representando cortes coronais encéfalo em caso experimental com 90 dias do grupo exposto a dose de 60 mg/kg de ibuprofeno. Em A corte total. Em B, C, D, E, F, G porção do hipotálamo. Contornos confeccionados e exportados a partir programa CorelDRAW® graphics suite X3 para serem utilizados na reconstrução tridimensional. A barra de calibração em A é válida para todas as imagens. VL: ventrículo lateral, 3V: 3º ventrículo, ac: comissura anterior, och: quiasma óptico.

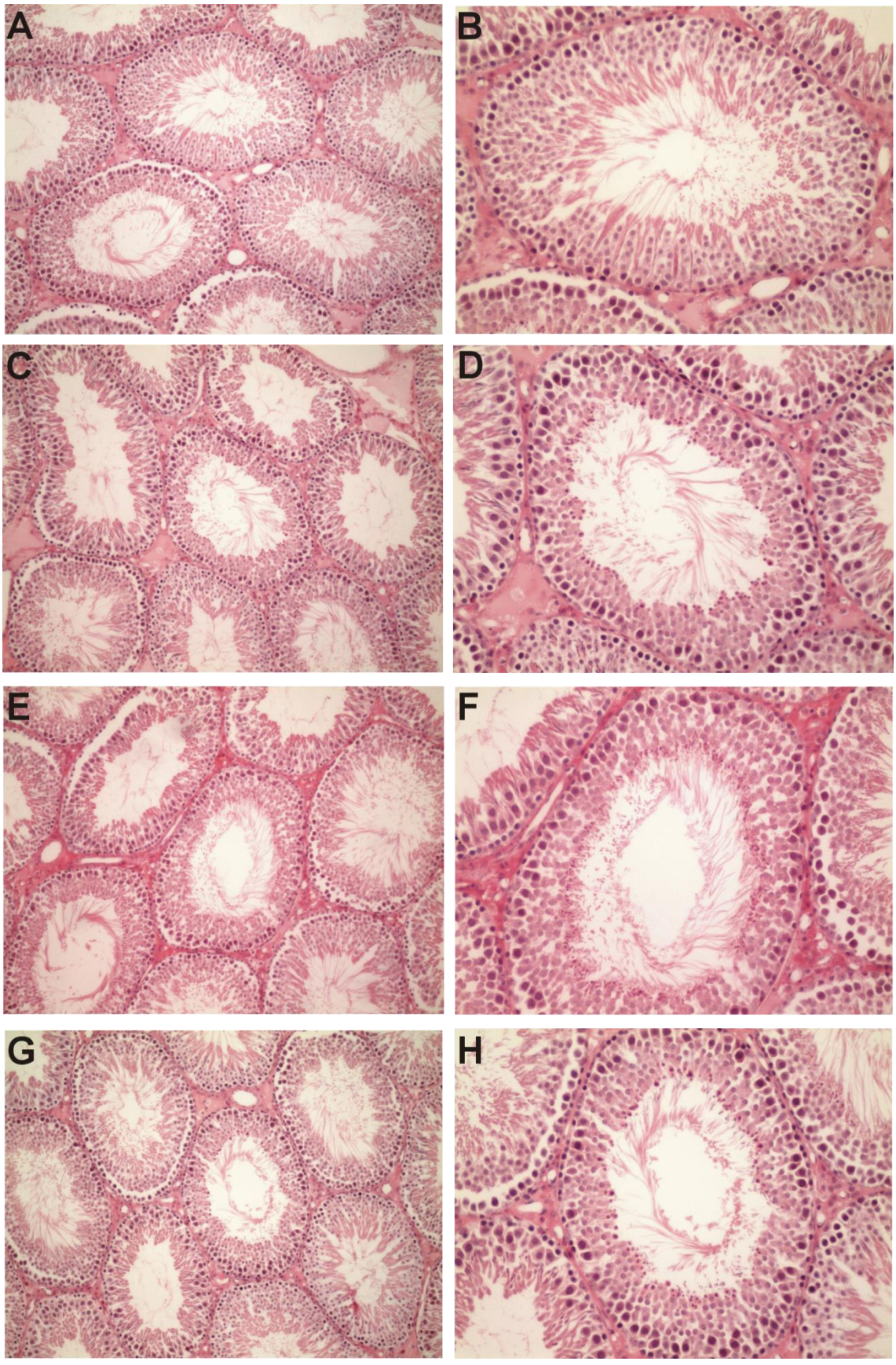

Figura 7 - Fotomicrografias de cortes transversais de testículo de ratos adultos no estágio VIII da espermatogênese. (A-B) Grupo Controle, (C-D) Grupo tratado com 10 mg/kg/dia de ibuprofeno, (E-F) Grupo tratado com 30 mg/kg/dia de ibuprofeno, (G-H) Grupo tratado com 60 mg/kg/dia de ibuprofeno. Coloração em HE. 10x e 20x.

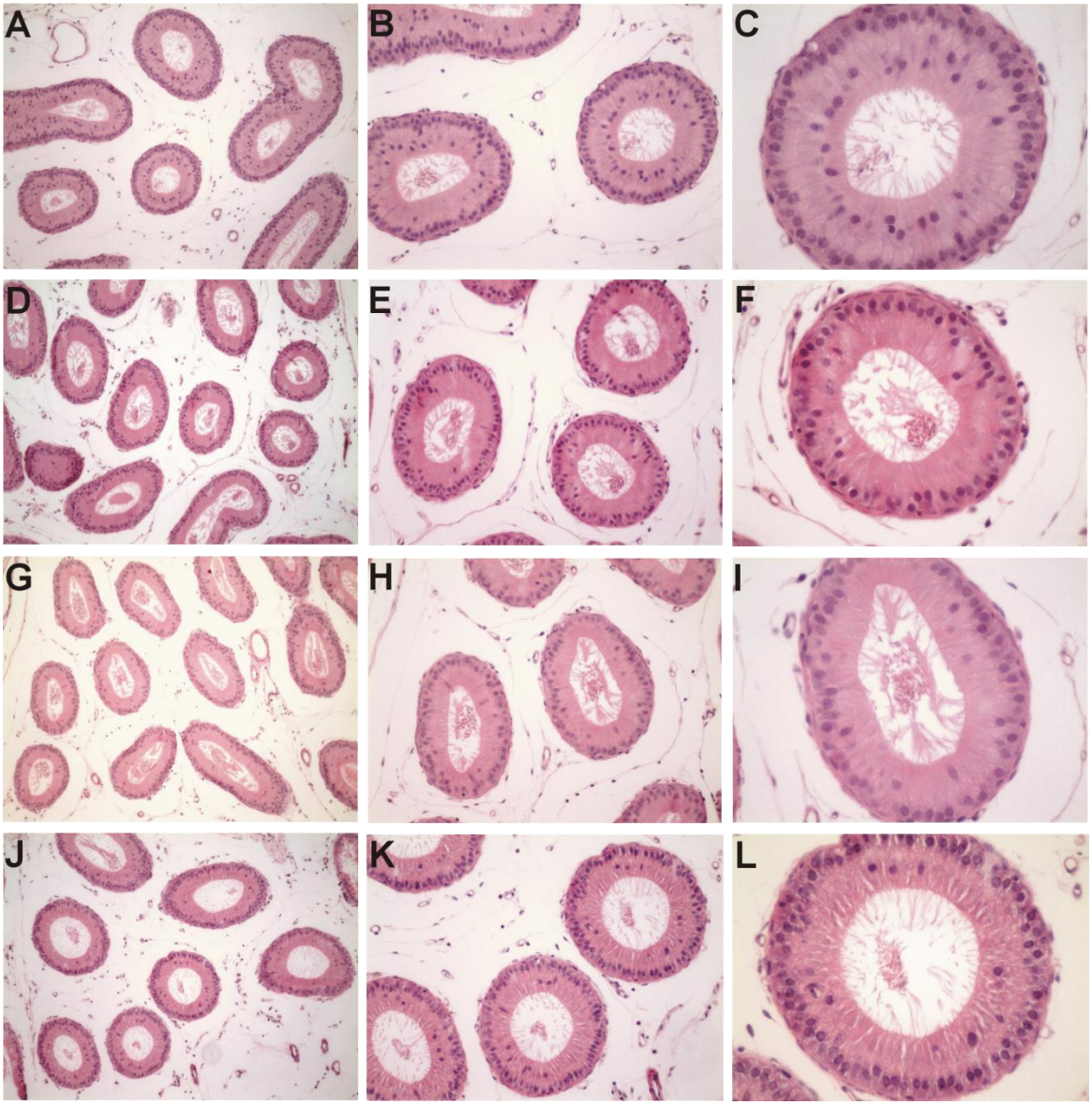

Figura 8 - Fotomicrografias de cortes longitudinais do segmento inicial do epidídimo de ratos adultos. (A-C) Grupo Controle, (D-F) Grupo tratado com 10 mg/kg/dia de ibuprofeno,(G-I) Grupo tratado com 30 mg/kg/dia de ibuprofeno, (J-L) Grupo tratado com 60 mg/kg/dia de ibuprofeno. Coloração em HE. 10x, 20x e 40x.

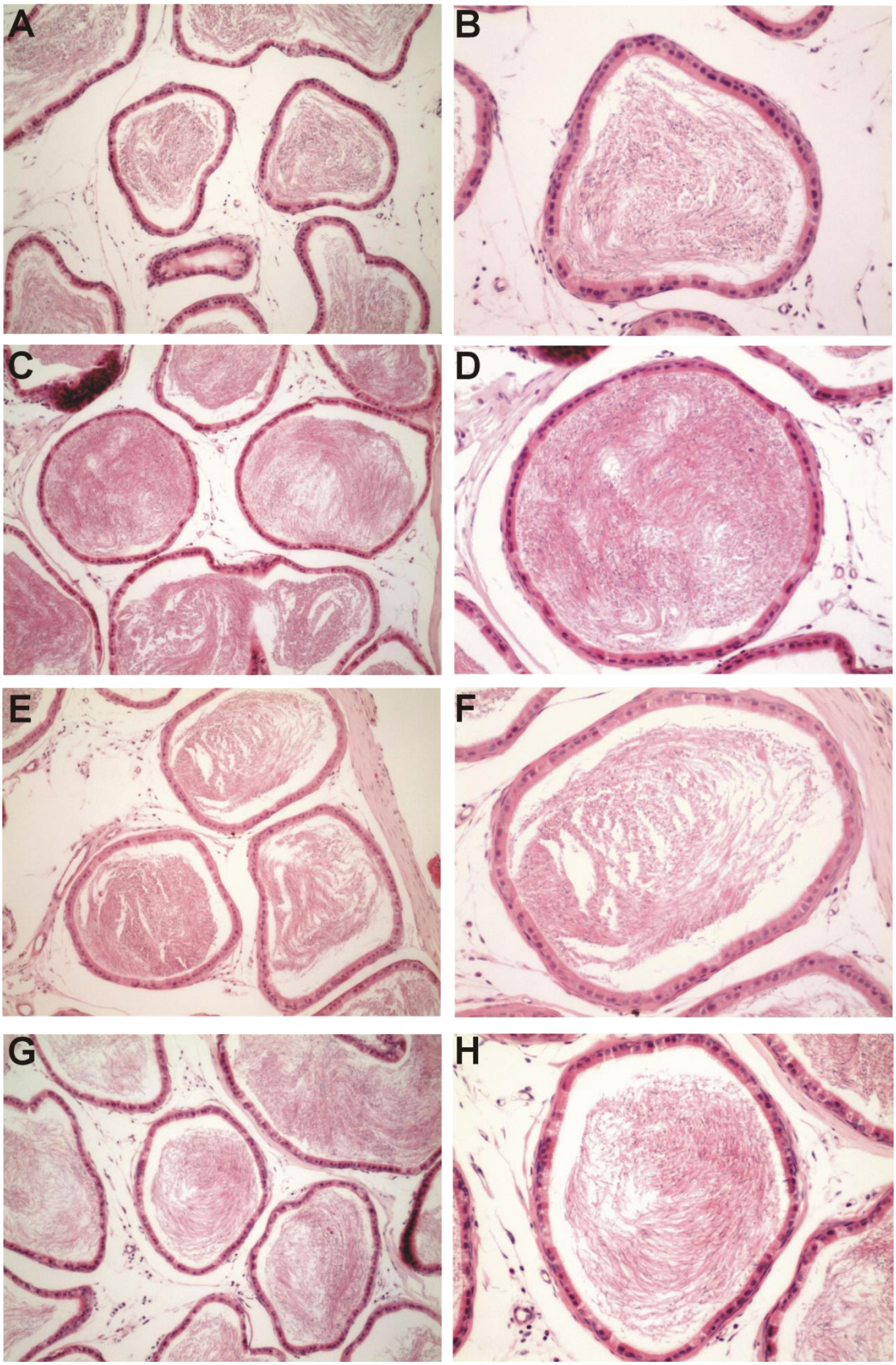

Figura 7 - Fotomicrografias de cortes longitudinais da região da cauda do epidídimo de ratos adultos. (A-B) Grupo Controle, (C-D) Grupo tratado com 10 mg/kg/dia de ibuprofeno, (E-F) Grupo tratado com 30 mg/kg/dia de ibuprofeno, (G-H) Grupo tratado com 60 mg/kg/dia de ibuprofeno. Coloração em HE. 10x e 20x.

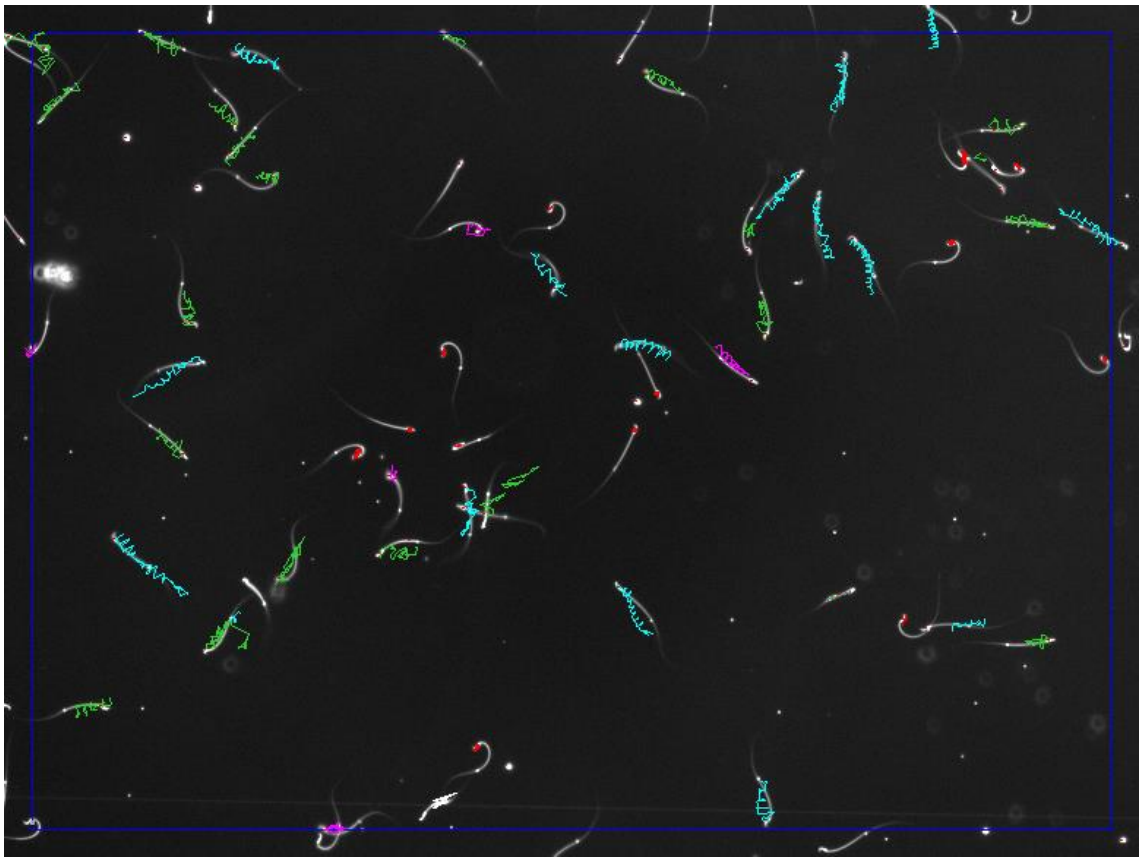

Figura 8 - Representação esquemática dos diferentes padrões de motilidade de espermatozoides do epidídimo de ratos adultos, conforme classificado pelo sistema CEROS II CASA. As faixas coloridas representam os espermatozoides móveis (verde), progressivos (azul), lentos (rosa) e estáticos (vermelho).

Comissão de Ética

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

Instituto de Biociências de Botucatu - Unesp

Certificado

Certificamos que o projeto intitulado "Influência da exposição perinatal ao anti-inflamatório ibuprofeno: repercussão tardia em parâmetros reprodutivos masculinos, em ratos", Protocolo nº **830-CEUA**, sob a responsabilidade de **Arielle Cristina Arena**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 9 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Vigência do Projeto:	*Início: 02/05/2016*	*Término: 23/2/2018*
Espécie/linhagem:	*Rato Wistar*	
Nº de animais:	*250*	
Peso:	*300g*	*Idade: 90 dias*
Sexo:	*Macho e fêmea*	
Origem	*Biotério Central da Unesp – Câmpus de Botucatu/SP*	

Botucatu, 18 de março de 2016.

Comissão de Ética no Uso de Animais IBB-Unesp

Prof. Adj. Wellerson Rodrigo Scarano
Presidente da CEUA

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