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IMPACTO DA UTILIZAÇÃO CRÔNICA DE CAFEÍNA NO PERÍODO GESTACIONAL E NEONATAL EM RATOS WISTAR: PARÂMETROS COMPORTAMENTAIS E NEUROQUÍMICOS

ANA CLÁUDIA DE SOUZA

Orientadora: Prof. Dra. Iraci Lucena da Silva Torres

DISSERTAÇÃO DE MESTRADO

Porto Alegre, janeiro de 2013.

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PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

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Dissertação apresentada ao Programa de Pós-Graduação em Medicina: Ciências Médicas, UFRGS, como requisito para obtenção do título de Mestre.

Porto Alegre, janeiro de 2013.

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“Mas a sabedoria que do alto vem é, primeiramente, pura, depois pacífica, moderada, tratável, cheia de misericórdia e de bons frutos, sem parcialidade e sem hipocrisia”.

Tiago 3:17

Resumo

Introdução: Estudos em animais e em humanos demonstram que o alto consumo de cafeína pode promover riscos e complicações obstétricas podendo resultar em eventos teratogênicos na prole, como alterações esqueléticas, retardo no crescimento intrauterino e baixo peso ao nascer e a partos prematuros. Embora a relação entre o consumo de cafeína durante a gravidez e seus efeitos tóxicos sobre o desenvolvimento embrionário venham sendo alvo de vários estudos, seus mecanismos ainda não estão totalmente esclarecidos. **Objetivos:** avaliar os efeitos da utilização crônica de cafeína no período gestacional e neonatal sobre o desenvolvimento da prole, reflexos motores, comportamentos, resposta nociceptiva, atividade e expressão em hipocampo da acetilcolinesterase e, atividade de ectonucleotidases em medula espinhal de ratos. **Métodos:** o ciclo estral das ratas com aproximadamente 90 dias (peso 200-300 g) foi avaliado por meio de esfregaço de lavado vaginal. Sendo a prenhez confirmada, as ratas foram divididas em 3 grupos experimentais: (1) controle; (2) cafeína; e (3) abstido de cafeína. As ratas controles recebiam somente água, as do grupo cafeína recebiam 0,3 g/L de solução de cafeína diluída em água e as abstidas recebiam a mesma solução até o 7º dia de vida pós-natal (P7) da prole, sendo após substituída por água. O dia do nascimento foi considerado P0, padronizou-se 8 machos por ninhada. O comportamento de endireitamento postural e resposta a geotáxia negativa foram avaliados do P1 até P14 e utilizados como parâmetros dos reflexos motores da prole. No final do tratamento (P14) foram avaliados: (1) o limiar nociceptivo, utilizando aparelho de *tail-flick;* (2) a atividade locomotora no campo aberto (3) a funcionalidade dos receptores de adenosina, por meio da administração de agonistas e antagonistas adenosinérgicos; (4) hidrólise de nucleotídeos em sinaptossomas de medula espinhal; e (5) a atividade e a expressão gênica da enzima acetilcolinesterase em hipocampo. Os dados foram expressos em Média±Erro Padrão da Média (EPM), e analisados utilizando ANOVA de uma via/Tukey e ANOVA de duas vias de medidas repetidas/Tukey de acordo com cada experimento, as diferenças foram consideradas significativas quando $P<0,05$. **Resultados:** os animais dos grupos cafeína e abstido apresentaram um atraso no desenvolvimento dos reflexos neurológicos ao longo dos 14 dias de vida quando comparados ao grupo controle. No campo aberto, os animais do grupo cafeína apresentaram diminuição no tempo total de locomoção e cruzamentos externos em relação ao controle. O grupo abstido aumentou cruzamentos internos e diminuiu rearing. No teste de *tail-flick*, não observou-se diferença entre os grupos. Apesar de não haver diferença entre os grupos na resposta nociceptiva basal no teste de tail-flick, quando avaliamos a funcionalidade dos receptores de adenosina do tipo A_1, observou-se que o grupo cafeína teve sua resposta diminuída ao agonista adenosinérgico (CPA) e o grupo abstido apresentou este efeito parcialmente revertido, demonstrando não ser um efeito de longa duração. No entanto, não foram observadas diferenças entre os grupos nas atividades de NTPDases e de 5'nucleotidase. Adicionalmente, observamos uma diminuição significativa na atividade da acetilcolinesterase (AChE) em hipocampo dos animais cafeínados comparados aos controles sem diferenças em sua expressão gênica, sugerindo assim que a baixa atividade desta enzima esta relacionada a mudanças pós-traducionais. **Conclusão:** Nossos resultados demonstram que o uso de cafeína durante a gestação e lactação pode trazer prejuízos ao desenvolvimento da prole, salientando a importância da restrição de alimentos e preparações que contenham esta substancia durante estes períodos.

Palavras-chave: Cafeína, período gestacional, comportamento, adenosina, acetilcolina, nocicepção.

Abstract

Introduction: Studies in animals and humans show that high consumption of caffeine can promote risks and obstetric complications may result in teratogenic events in the offspring, such as skeletal abnormalities, intrauterine growth retardation and low birth weight and premature births. Although the relationship between caffeine consumption during pregnancy and its toxic effects on embryonic development may be the target of several studies, its mechanisms are not fully understood. **Objectives:** the aim was to evaluate the effects of gestational chronic caffeine intake and neonatal offspring development, neurological reflexes, behavior, nociceptive response, acetylcholinesterase activity and expression in hippocampus and ectonucleotidases activity in spinal cord of rats. **Methods:** adult female Wistar rats were performed vaginal lavage to verify the estrous cycle. Mating was confirmed by sperm presence in vaginal smears. On the first day of pregnancy, rats were divided into three groups: (1) control, (2) caffeine and (3) washout. Control animals received only water; caffeine group received caffeine solution 0.3 g / L diluted with water and the washout group received the same caffeine solution until the 7th day (P7), which was replaced by water. The birth date was considered P0, animals were standardized at 8 male animals per group. The righting reflex (RR) and negative geotaxis (NG) behaviors were measured from P0 to P14 and were using as motor reflexes. At P14 were evaluated: (1) the nociceptive response by tail-flick latency (TFL); (2) locomotor activity by open field test (OF); (3) functionality of A1 adenosine receptors by agonist and an antagonist administration of DPCPX and CPA; (4) nucleotides and nucleoside hydrolysis by spinal cord synaptosomes and (5) acetylcholinesterase (AChE) activity and gene expression in hippocampus. Data were expressed as mean ± SEM and analyzed using one-way ANOVA / Tukey and repeated measures ANOVA/Tukey, values were considered significant if $P<0.05$. **Results:** caffeine and washout groups presented a delay in the development of neurological reflexes over the 14 days of life when compared to the control group. In the open field, caffeine group showed a decrease in the total time of locomotion and outer crossing in relation to control. The washout group presented increase in inner crossing and decrease in rearing behavior. In the tail-flick test, no difference was observed between groups. Although there is no difference between the groups in baseline nociceptive response in the tail-flick test, when evaluating the functionality of A1 adenosine receptors, caffeine group presented a decreased response to adenosinergic agonists (CPA) and the washout group presented partially reversed of this effect, demonstrating it is not a long duration effect. However, no differences were observed between groups in the activities of NTPDase and 5'nucleotidase. Additionally, we observed a significant decrease in the activity of acetylcholinesterase in hippocampus of caffeine group compared to controls without differences in their gene expression, suggesting that the low activity of this enzyme is related to post-translational changes. **Conclusion:** Our results demonstrate that caffeine intake during pregnancy and lactation can bring harm to the offspring developing. Therefore, it becomes increasingly important restriction of food and caffeine-containing preparations during the embryonic period.

Keywords: Caffeine, gestation, adenosine, acetylcholine, behavior, nociception.

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Lista de Abreviaturas

ACh	Acetilcolina
AChE	Acetilcolinesterase
ADO	Adenosina
ADP	Adenosina 5’ difosfato
AMP	Adenosina 5’ monofosfato
ANOVA	Análise de Variância (Analysis of Variance, em inglês)
ARs	Receptores de adenosina
ATP	Adenosina 5’ trifosfato
cAMP	Adenosina 3',5'-monofosfato cíclica
CA^{+}	Íon cálcio
CPA	N6-Cyclopentyladenosine
DA	Dopamina
DARPP-32	Dopamina e fosfoproteína regulada por cAMP neuronal
EDTA	Ácido etilenodiaminotetracético
(E-)NDPK	(Ecto-)nucleosídeo difosfoquinase
(E-)NPP	(Ecto-)nucleotídeo pirofosfato/fosfodiesterase
(E-)NTPDASE	(Ecto-)nucleosídeo trifosfato difosfo-hidrolase
EPM	Erro padrão da média
GABA	Ácido-γ-aminobutírico
GABAR	Receptores GABA
K^{+}	Íons potássio
KCl	Cloreto de potássio
mg	Miligrana

µL	Microlitro
M	Molar
Na^-	Íon sódio
NMDA	N-metil-D-aspartato
P0	Dia do nascimento
P7	7° dia pós-natal
P14	14º dia pós-natal
A_1	Receptor adenosinérgico
A_{2A}	Receptor adenosinérgico
A_{2B}	Receptor adenosinérgico
A_3	Receptor adenosinérgico
P1	Receptor purinérgico de adenosina
P2	Receptor purinérgico de ATP
RNA	Ácido Ribonucleico
RNAm	RNA mensageiro
SNK	Student-Newman-Keuls
SNC	Sistema Nervoso Central
THR34	Treonina 34

SUMÁRIO

1 INTRODUÇÃO

A cafeína é considerada a substância psicoativa mais consumida mundialmente (Tavares e Sacata, 2012). Os efeitos agudos e crônicos da cafeína podem ser bastante diferentes. Mas a maioria dos efeitos centrais em concentrações encontradas nas bebidas é devido ao bloqueio dos receptores de adenosina (Ribeiro e Sebastião, 2010). O uso de cafeína é capaz de promover efeitos em inúmeras funções fisiológicas, como resistência física, humor, sono e dor (Fredholm *et. al.*, 1999).

Além do seu consumo em alimentos e bebidas (chocolates, cafés, chás, bebidas de cola, entre outros), é importante destacar o uso de cafeína em medicamentos e fórmulas farmacêuticas. Entre as utilizações terapêuticas da cafeína destaca-se seu uso como estimulante do sistema nervoso central (SNC) (Zhang *et al*., 2011) e tratamento de apneia em neonatos prematuros, em que é utilizada por via endovenosa como citrato de cafeína (Schmidt *et al*., 2006). Quando associada à benzoato de sódio, é utilizada como estimulante ou para tratamento da cefaleia após anestesia subaracnóidea (Tavares & Sacata, 2012). Além disto, é comumente associada a analgésicos e anti-inflamatórios principalmente para o tratamento da cefaleia (Derry *et al*., 2012).

A cafeína é uma substância de potencial abuso (Gilliland & Bullock, 1984; Holtzman, 1990; Juliano e Griffiths, 2004; Tavares & Sacata, 2012) e seu intenso uso tem levado a um aumento do número de estudos que abordam a avaliação dos efeitos da exposição crônica sobre o feto e neonatos (Li *et al*., 2012). Em torno de 95% das mulheres em idade reprodutiva consomem cafeína regularmente, e destas 72% mantem o uso durante a gravidez e amamentação (James, 1991).

A cafeína é capaz de atravessar facilmente a barreira placentária (Fredholm *et al*., 1999), devido a sua lipossolubilidade, no entanto o feto é incapaz de metabolizá-la, pois neste período seus sistemas enzimáticos hepáticos encontram-se imaturos (Knutti *et al*., 1982; Soellner *et al*., 2009). É interessante observar que, em mulheres grávidas, especialmente no segundo e terceiro trimestres de gestação, a meia-vida da cafeína aumenta para sete e dez horas, respectivamente, enquanto que em mulheres não grávidas a depuração da cafeína varia entre duas horas e meia a quatro horas e meia (Knutti *et al*., 1982; Tracy *et al*., 2005, Palmer *et al*., 2010).

Estudos em animais e em humanos demonstram que o alto consumo de cafeína pode acarretar riscos e complicações obstétricas podendo resultar em eventos teratogênicos na prole (Klebanoff *et al*., 1999; Cnattingius *et al*., 2000; Tavares & Sacata, 2012), como alterações esqueléticas, retardo no crescimento intrauterino e baixo peso ao nascer (Sawynok & Yaksh, 1993). Embora a relação entre o consumo de cafeína durante a gravidez e seus efeitos tóxicos sobre o desenvolvimento embrionário vem sendo alvos de vários estudos, seus mecanismos ainda não estão totalmente elucidados (Li *et al*., 2012).

É sabido que a cafeína é utilizada frequentemente para induzir a vigília e diminuir a fadiga (Heckman *et al.,* 2010). Esta ação da cafeína está ligada a sua afinidade pelos receptores de adenosina A_1 e A_{2A}, atuando como um antagonista. Estudos em camundongos nocautes demostraram que o receptor A_{2A} está relacionado ao estado de alerta, enquanto o receptor A_1 está ligado ao estado de vigília (Huang *et al*., 2005). Além disto, a adenosina (ADO) endógena regula e modula a transmissão da dor em nível periférico, espinhal e supra-espinhal (Sawynok, 1998), sendo sua ação dependente do local de administração e do receptor adenosinérgico ativado, tendo um efeito modulatório na resposta nociceptiva (Sawynok, 1998; Sawynok & Liu, 2003).

Estudos demonstram que a administração de ADO e de seus análogos, via central ou sistêmica, induz a antinocicepção em uma variedade de modelos animais (Sawynok, 1998; Lavand'homme & Eisenach, 1999; Gong *et al*., 2010).

Outro importante sistema modulatório a ser considerado é o sistema colinérgico. Pedata e colaboradores (1984) demonstraram que uma dose de cafeína 50 µM produz aumento na liberação de acetilcolina (ACh) em fatias de córtex cerebral de ratos e este efeito também foi observado na dose de 100 µM em sinaptossoma de hipocampo de ratos, importante estrutura envolvida com aprendizagem, memória (Blozovski, 1985; Izquierdo *et al*., 2008) e cognição (Hurst *et al*., 2012). A ACh atua como um neuromodulador do SNC (para revisão ver Yakel, *in press*), tem um papel fundamental como neurotransmissor periférico excitatório, e contribui para a plasticidade sináptica em muitas áreas do cérebro (para revisão ver Yakel, *in press*). A ACh está envolvida na ativação cortical, atenção, aprendizado, memória, dor, controle do motor e controle das funções autonômicas (Herlenius & Lagercrantz, 2004).

A exposição precoce a substâncias psicoestimulantes pode induzir ao fenômeno de "imprinting neural", onde a substância pode ter efeitos não necessariamente imediatos, mas manifestando-os mais tardiamente (Andersen, 2005). Essa plasticidade neuronal é decorrente da notável característica do sistema nervoso ser maleável frente a estímulos provenientes do meio externo. Estas alterações plásticas são observadas durante o desenvolvimento, no processo de aprendizado e memória e em resposta a estímulos externos ou doenças (Lledo et al., 2006). Estudos tem demonstrado que a exposição à cafeína no início do desenvolvimento neural está relacionada a alterações comportamentais e nociceptivas que podem ser de longa duração permanecendo até a vida adulta (Andersen, 2005, Feldman et al., 1997; Daly & Fredholm, 1978).

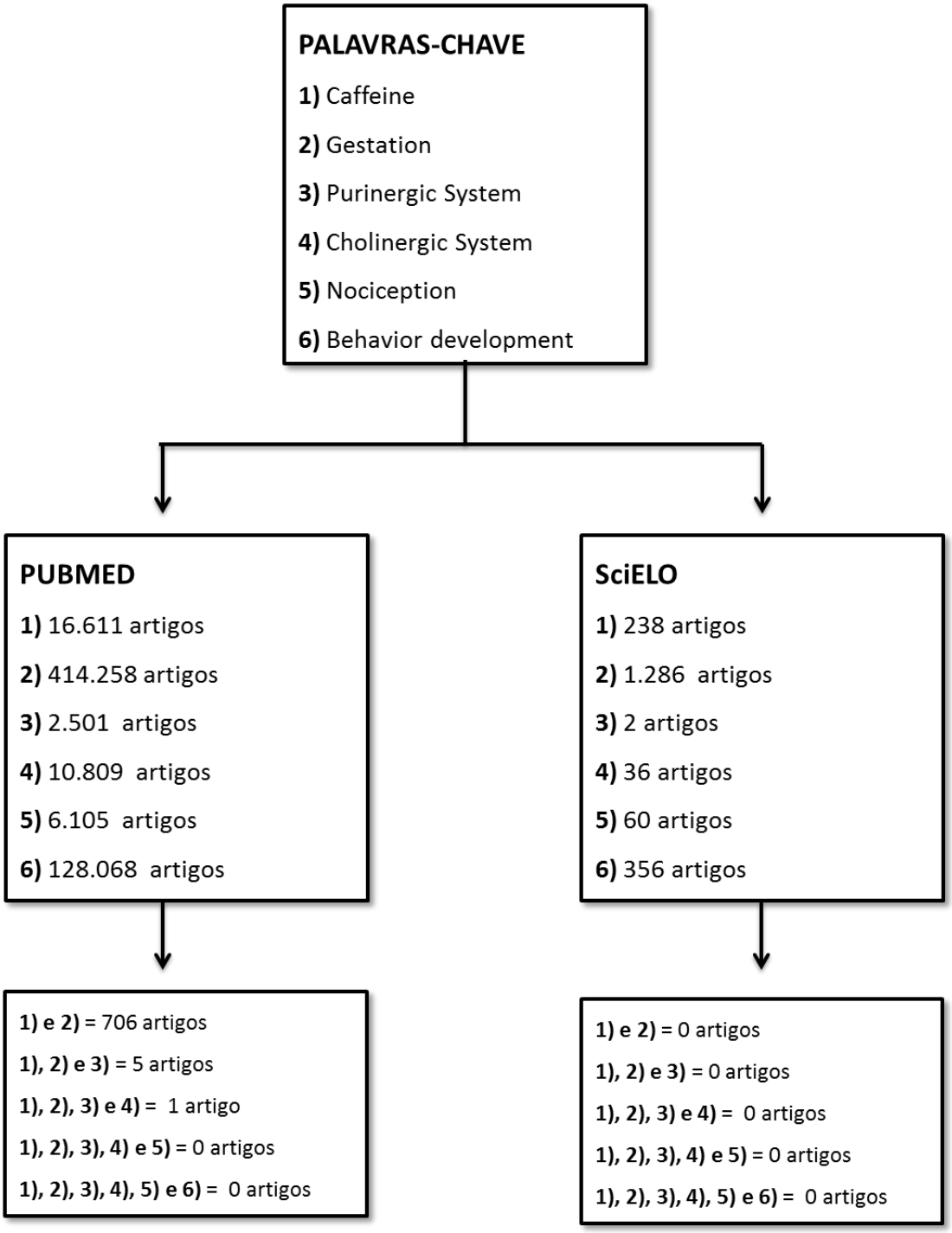
Considerando o que foi exposto, se faz necessário o aprofundamento de estudos que abordem diferentes sistemas suscetíveis aos efeitos da exposição crônica a cafeína, entre eles, os sistemas colinérgico e purinérgico. A utilização de modelos animais, como o proposto nesta dissertação, permite avaliar os efeitos da cafeína no desenvolvimento dos filhotes, bem como alterações comportamentais e neuroquímicas.

2 ESTRATÉGIAS PARA LOCALIZAR E SELECIONAR INFORMAÇÕES

Na revisão de literatura buscou-se apresentar os principais aspectos do uso de cafeína, gestação, sistema purinérgico, sistema colinérgico, nocicepção e desenvolvimento comportamental. A estratégia de busca envolveu as seguintes bases de dados: MEDLINE (site PubMed) e SciELO. Foram selecionados artigos publicados entre 1990 e 2013.

Nos sites PubMed e SciELO foram realizadas buscas utilizando os termos*: Caffeine,gestation, purinergic system, cholinergic system, nociception and behaviour development.* Em relação ao termo *caffeine,* foram encontrados 16.611 artigos no PubMed e 238 artigos no SciELO. Utilizando-se o termo: *gestation* foram encontrados 414.258 artigos no PubMed e 1.286 no SciELO. Com o descritor *Purinergic System* a busca no PubMed encontrou 2.501 artigos e apenas 2 artigos no SciELO. Com o descritor *Cholinergic System* o site PubMed identificou 10.809 artigos e no SciElo 36 artigos. Para *Nociception,* foram encontrados 6.105 no PubMed e 60 no SciELO. Em relação à *behavior development,* 128.068 artigos foram encontrados no PubMed e 356 no SciELO. Refinando-se a busca, com cruzamentos entre as palavras-chave foi encontrado um reduzido número de artigos como mostrado na Figura 1.

Figura 1. Fluxograma da pesquisa realizada sobre o tema.



(Fonte: do autor)

3 REVISÃO DA LITERATURA

3.1 Cafeína

A cafeína têm sua origem a partir da descoberta do café (1258 d.C.) e do chá (2737 a.C.) (Fredholm, 2011; Mair & Hoh, 2009). Os grãos de café, primeiramente eram comidos, e mais tarde, moídos e misturados com uma pasta de gordura como um lanche estimulante de viagem (Fredholm, 2011). Por volta do ano 1000 d.C., as infusões de café com água fervente começaram a ser produzidas. Por volta do século XIV, o processo de torrefação foi desenvolvido, e seu uso se espalhou rapidamente.

A palavra "café" vem de qahva (ou qahwah) que denota de uma bebida feita a partir de plantas. A cidade de Mocha se tornou o centro do café – e seu nome designa "beber". Hoje o café é amplamente cultivado em mais de 50 países (Ukers, 1922; Weinberg & Bealer, 2001; Fredholm, 2011).

Inicialmente o uso de cafeína era destinado a fins medicinais (Ukers, 1922; Weinberg & Bealer, 2001; Mair & Hoh, 2009). Em meados do século XIX surgiram relatos sobre necessidade de estudos que embasassem cientificamente os efeitos da cafeína. O químico Friedrich Ferdinand Runge (1795-1865) foi o primeiro que identificou em sua forma pura a substância que hoje chamamos de cafeína (Weinberg & Bealer, 2001). O estudo da química da cafeína foi desenvolvido por Hermann Emil Fischer (1852-1919). Cole em 1833, relatou sobre os efeitos negativos de altas doses de cafeína. Mais tarde Samuel Hahnemann (1755-1843), escreveu sobre os efeitos

benéficos da cafeína na atenção e alerta, mas advertiu contra o seu uso, por envolver o rompimento de um equilíbrio natural (Hahnemann, 1803).

A cafeína está presente em grande variedade de alimentos e bebidas, como cafés, chás, chocolates e derivados da cola em diferentes concentrações (Tabela 1) (Sawynok & Yaksh, 1993; Fredholm *et al*., 1999; Kuczkowski, 2009a,b,c), refletindo um alto consumo de cafeína, inclusive durante a gravidez (Gilbert, 1984).

Tabela 1. Teor de cafeína de vários alimentos e bebidas

Produto	Volume ou Peso	Teor de Cafeína (mg)
Café torrado e moído		
Percolado	150 ml	40-170
Pingado	150 ml	60-180
Descafeinado	150 ml	2-5
Café instantâneo		
Cafeinado	150 ml	40-180
Descafeinado	150 ml	2-8
Barra de Chocolate		
Ao leite	28 g	1-15
Amargo	28 g	5-35
Refrigerantes		
Cola	180 ml	15-24
Cola Diet	180 ml	13-29

(Fonte: Adaptado da Revisão de Fredholm *et al*., 1999)

Estudos iniciais indicaram que o consumo de cafeína durante a gestação estaria relacionado ao baixo peso ao nascer, anormalidades esqueléticas e diminuição do crescimento (Fernandes *et al*., 1998; Santos *et al*., 1998). Em 1980, com base em

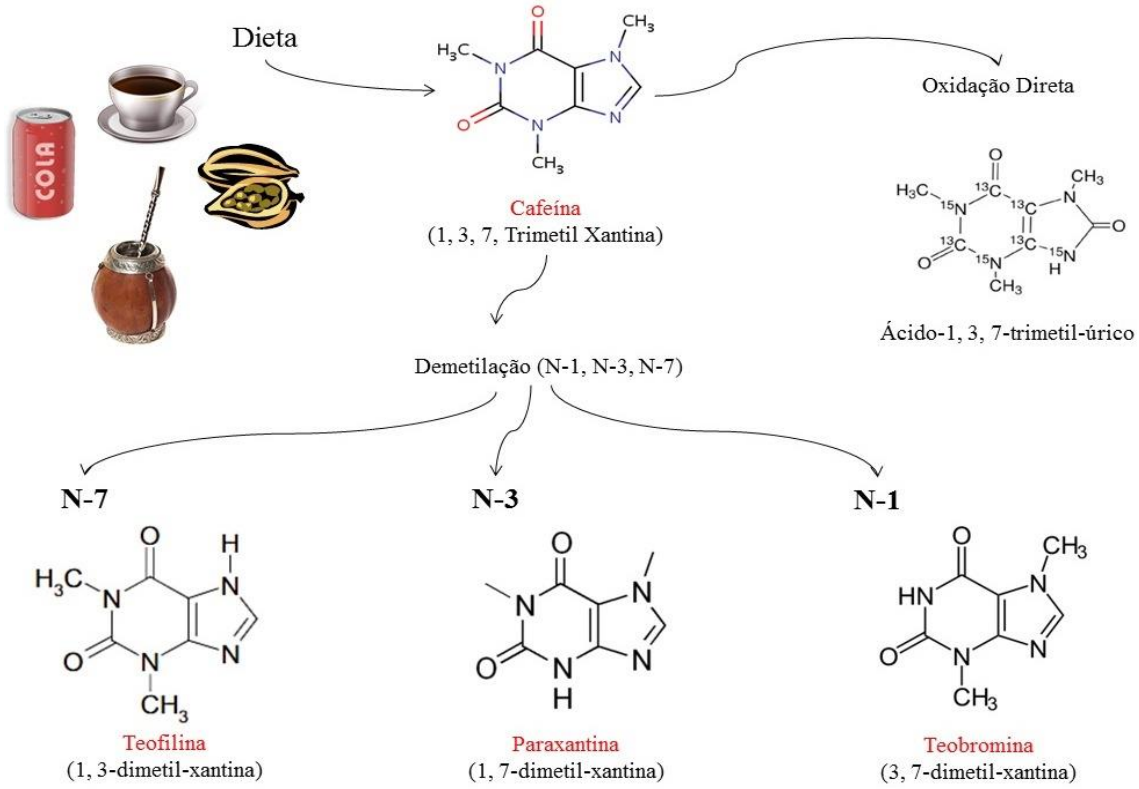
estudos animais, a *Food and Drug Administration* (FDA) sugeriu que as mulheres grávidas evitassem ou diminuíssem o consumo de alimentos e/ou bebidas contendo cafeína (Fredholm, 2011). O consumo de altas doses de cafeína, durante a gestação, está associado a partos prematuros (Khanna & Somani, 1984).

Em neonatos, a cafeína tem sua meia-vida aumentada devido à menor atividade da citocromo P-450 (Aranda *et al*., 1979) e imaturidade de algumas vias de desmetilação e acetilação (Aranda *et al*, 1974). No recém-nascido a termo a meia-vida da cafeína é de cerca de 80 $\pm$ 23 h (Aranda *et al*, 1977;. Le Guennec & Billon, 1987) e podendo ser superior a 100 h em prematuros (Parsons & Neims, 1981). Após os 3 meses de idade, a meia-vida da cafeína começa a diminuir exponencialmente (Aldridge *et al.*, 1979;. Parsons & Neims, 1981; Pearlman *et al.*, 1989).

A cafeína é rápida e totalmente absorvida pelo trato gastrointestinal tanto em humanos quanto em ratos (Arnaud, 2011), devido a sua lipossolubilidade atravessa todas as barreiras biológicas (Lachance *et al*., 1983; Tanaka *et al*., 1984), inclusive a placentária (Ikeda *et al*.; 1982; Kimmel *et al*., 1984).

A cafeína tem metabolização hepática (Arnaud, 2011), sendo que a principal diferença entre roedores e humanos é o fato de que, no rato, 40% dos metabolitos são derivados trimetil em comparação com menos de 6% em seres humanos (Arnaud, 2011). No humano a metabolização é caracterizada quantativamente pela desmetilação de 3-metil levando à formação de paraxantina, o que representa 72 a 80% do metabolismo da cafeína (Figura 2) (Arnaud *et al.*, 1982). Muitas das etapas do metabolismo da cafeína podem ser saturáveis em seres humanos, dependendo da dose, como por exemplo, a eliminação de alguns dos seus metabolitos (Kaplan *et al*., 1997).

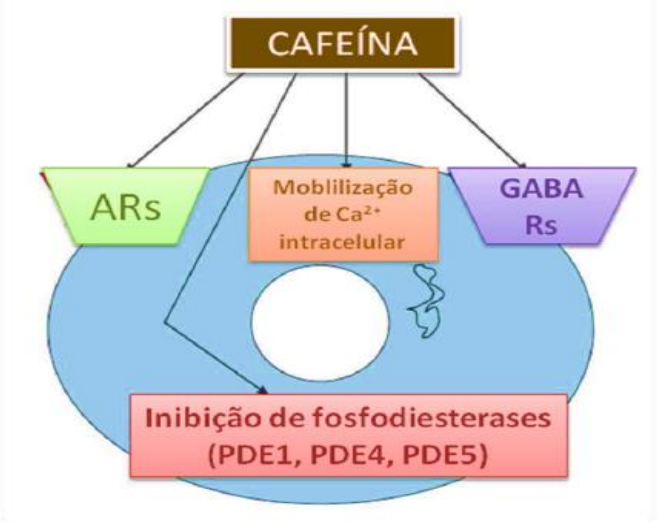
Figura 2. Biotransformação da cafeína.



A cafeína é absorvida a partir da dieta, pode sofrer oxidação direta formando ácido-1,3,7-trimetil-úrico, ou demetilação em N-7, N-3 e N-1 levando a formação de teofilina, paraxantina e teobromina, respectivamente. (Fonte: do autor).

A cafeína tem ação estimulante do sistema nervoso central (SNC), seu mecanismo de ação está relacionado com (1) antagonismo de receptores adenosinérgicos, (2) inibição de receptores $GABA_{A,}$ (3) ativação de receptores rianodina, um canal liberador de Ca+ intracelular, (4) inibição dos efeitos das fosfodiesterases, aumento do influxo de Ca^{+} e do efluxo de K^{+}, aumento de AMP-cíclico e inibição de atividade lipolítica, resultando em aumento do efeito e da duração do AMPc intracelular com consequente potencialização dos efeitos e da liberação de catecolaminas (Figura 3) (Shi *et al*., 2003; Sawynok, 2011, Castro *et al*., 2005).

Figura 3. Mecanismos de ação da cafeína.



(Fonte: Adaptado de Ribeiro & Sebastião, 2010). ARs: Receptores de Adenosina; PDE: fosfodiesterases.

3.1.2 Cafeína e Sistema Purinérgico

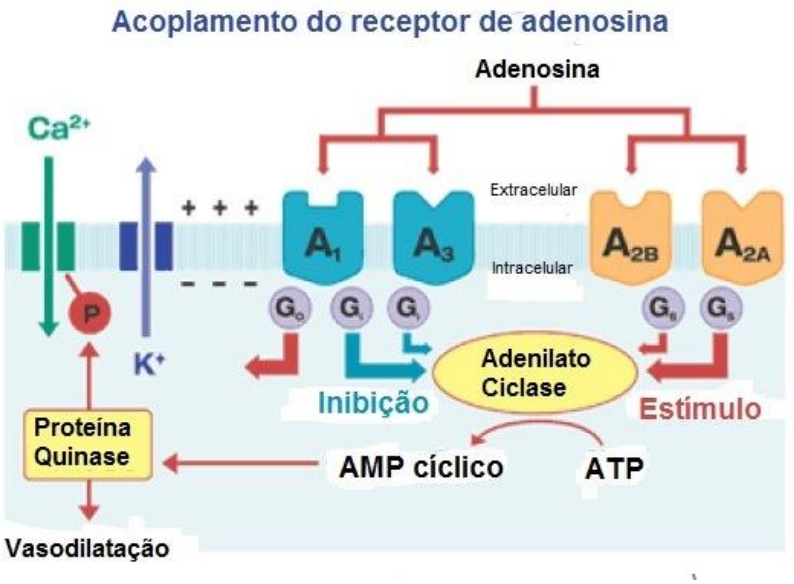
A cafeína é utilizada frequentemente para induzir a vigília e diminuir a fadiga (Heckman *et al.*, 2010). Esta ação da cafeína está ligada a sua afinidade pelos receptores de adenosina A_1 e A_{2A}. Estudos em camundongos nocautes demostraram que o receptor A_{2A} está relacionado ao estado de alerta, enquanto o receptor A_1 está ligado ao estado de vigília (Huang *et al*., 2005). A adenosina é um neuromodulador envolvido na regulação sono-vigília, quando a cafeína se liga aos receptores adenosinérgicos, promove o bloqueio da ação da adenosina endógena. (Porkka-Heiskanen *et al*., 1997, Huang *et al*., 2011).

A adenosina atua via receptores P1 que são divididos em quatro subtipos A_1, A_{2A}, A_{2B}, A_3, todos acoplados à proteína G (Fredholm *et al*., 2001). Os receptores A_1 e A_{2A} exercem ações antagônicas sobre a transmissão neuronal, visto que os receptores A1 são acoplados a proteínas G inibitórias e os A_{2A} são acoplados a proteínas G

excitatória que estimulam a síntese de AMPc (Fredholm *et al*., 2005). A atividade de canais iônicos e o estado de fosforilação de proteínas são alvos intracelulares da sinalização adenosinérgica pelos quais a neurotransmissão é afetada.

Os receptores A_1 e A_3 de adenosina estão acoplados às proteínas Gi/Gq (G inibitória/G quinase) e os receptores A_2 de adenosina estão acoplados à proteína Gs (G estimulatória). P: fosfato; Ca^{2+}: cálcio; K^+: potássio; ATP: Adenosina Trifosfato (Figura 4).

Figura 4. Receptores adenosinérgicos.



(Fonte: Adaptado de http://www.aderis.com/img/art_adenosine.gif).

Por outro lado, adenosina 5’ trifosfato (ATP) e adenosina 5’ difosfato (ADP) atuam via receptores P2 que são divididos em dois subgrupos: P2X (ionotrópicos), subdivididos em P2X1 à P2X7 e P2Y (metabotrópicos), subdivididos em P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 e P2Y14 (Tabela 2).

Tabela 2. Classificação dos receptores purinérgicos.

	Receptores P1	Receptores P2	
Ligante natural	Adenosina	ATP, ADP, UTP e UDP	
Tipo	Acoplado a proteína G	Canal Iônico	Acoplado a proteína G
Subgrupo	-------	P2X	P2Y
Subtipo	A1, A2A, A2B e A3	P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 e P2X7	P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 e P2Y14

(Fonte: Adaptado de Ralevic e Burnstock, 1998)

Entre as ações exercidas pelos nucleotídeos e nucleosídeo da adenina estão: desenvolvimento neuronal, atividade locomotora, nocicepção, aprendizado e a memória (Pankratov, *et al*., 2002; Pedrazza, *et al*., 2007; Burnstock, 2006). A sinalização purinérgica depende da concentração extracelular de nucleotídeos e nucleosídeo e do tipo de receptor ativado por eles (Tabela 2) (Fredholm, *et al*., 2005).

O controle dos níveis extracelulares de nucleotídeos e nucleosídeo é realizado por enzimas localizadas na superfície celular e /ou secretadas no meio extracelular (solúveis) (Zimmermann, 2001), constituindo as famílias das enzimas ectonucleotídeo trifosfato difosfoidrolases (E-NTPDase), ectonucleotídeo pirofosfatase/fosfodiesterases (E-NPP), fosfatase alcalina e a ecto - 5'- nucleotidase (Zimmermann, 2001; Cunha, 2001).

As NTPDases são as principais enzimas envolvidas na degradação dos nucleotídeos purinérgicos e, juntamente com a ecto-5´-nucleotidase, promovem degradação sequencial do ATP até adenosina (Burnstock, 2013). A participação da ecto-

5’-nucleotidase na via das ectonucleotidases exerce um papel modulador sobre a produção de adenosina extracelular, sendo a enzima marca-passo desta cascata enzimática (Zimmermann, 1996; Cunha, 2001). A hidrólise dos nucleotídeos purinérgicos associada aos transportadores específicos de nucleosídeo e nucleotídeos constituem as formas de controle da disponibilidade destes para seus receptores específicos.

Os efeitos da administração crônica ou aguda de agonistas e antagonistas de receptores adenosinérgicos é uma importante ferramenta para elucidar o papel destes receptores no desenvolvimento neural (Guillet & Kellogg, 1991; Adén *et al*., 2001; Léon *et al*., 2002; Lipska *et al*., 2002, Turner *et al*., 2002). Alguns trabalhos avaliando o efeito de agonistas de receptores A_1 durante a fase de desenvolvimento neural demonstraram que a ativação destes receptores promove um efeito deletério sobre o desenvolvimento, o que se opõe ao efeito neuroprotetor clássico da adenosina (Rivkees et al., 2001; Turner et al., 2002). Sabe-se que a cafeína tem ação em receptores de adenosina, a administração crônica de cafeína no período de desenvolvimento e maturação do sistema adenosinérgico pode ser capaz de promover mudanças na funcionalidade destes receptores (Burnstock, 2007; Léon et al., 2002). Tão importante quanto o controle dos níveis de ADO extracelular, a ativação de receptores adenosinérgicos é de crucial importância para o estabelecimento de conexões neurais em cérebros imaturos (Zimmermann, 2006).

Outra importante ação da adenosina (ADO) endógena é sua participação da modulação da transmissão nociceptiva em nível periférico, espinhal e supra-espinhal (Sawynok, 1998) estando sua ação dependente do local de administração e do receptor adenosinérgico ativado (Sawynok, 1998; Sawynok & Liu, 2003). A ADO atua na resposta nociceptiva ativando receptores específicos nos diferentes níveis de atuação

(Burnstock & Wood, 1996; Sawynok, 1998; Gerevich & Illes, 2004). A liberação de ADO nos sítios espinhais tem sido demonstrada na presença de altas concentrações de K^+, de capsaicina, de substância P, de glutamato, de morfina, de serotonina e de inibidores da adenosina quinase (Sweeney *et al*., 1990; Cahill *et al*., 1993; Cahill *et al*., 1995; Cahill *et al*., 1997; Poon & Sawynok, 1999; Sandner-Kiesling *et al.*, 2001). Em sítios periféricos, a adenosina é liberada de terminações nervosas sensoriais em presença de capsaicina, de glutamato, de formalina, de inibidores da adenosina quinase e de inflamação local (Liu *et al*., 2001; Liu *et al*., 2002; Sawynok & Liu, 2003, Aumeerally, *et al*., 2004).

A ação da ADO na transmissão do estímulo doloroso exibe efeitos diversos dependentes do local e tipo de receptor ativado (Sawynok, 1998). A ADO pode atuar em neurônios espinhais, primariamente por ativação de receptores A_1, e em neurônios periféricos via receptores A_1 e A_{2A} (Taiwo & Levine, 1990; Aley *et al*., 1995; Lee & Yaksh, 1996; Poon & Sawynok, 1999, Ferre *et al*., 2008, Lorenzo, *et al*., 2010). A ativação de receptores A_1 localizados em neurônios da medula espinhal ou em interneurônios pode resultar em inibição pós-sináptica da transmissão excitatória, com consequente abertura de canais de K^+ hiperpolarizando a membrana celular (Sawynok & Liu, 2003). Já os receptores A_{2A} estão presentes em neurônios do gânglio da raiz dorsal e potencialmente presentes nos terminais pré-sinápticos espinhais dos neurônios sensoriais aferentes (Sawynok & Liu, 2003). O envolvimento dos receptores A_{2A} na transmissão espinhal do estímulo nociceptivo é menos conhecido, mas é sugerido que as ações supra-espinhais da morfina sejam mediadas por estes receptores, enquanto que a administração de endorfinas parece ter suas ações mediadas por receptores A_1 e A_2 (Sawynok, 1998).

Ainda que a ação primária de cafeína seja por bloqueio de adenosina, ocorrem diversos efeitos secundários em muitas classes de neurotransmissores, incluindo a noradrenalina (NA), dopamina (DA), serotonina (5-HT), acetilcolina, o glutamato, e GABA (Ácido Gama-Aminobutírico) (Daly *et al.*, 1993).

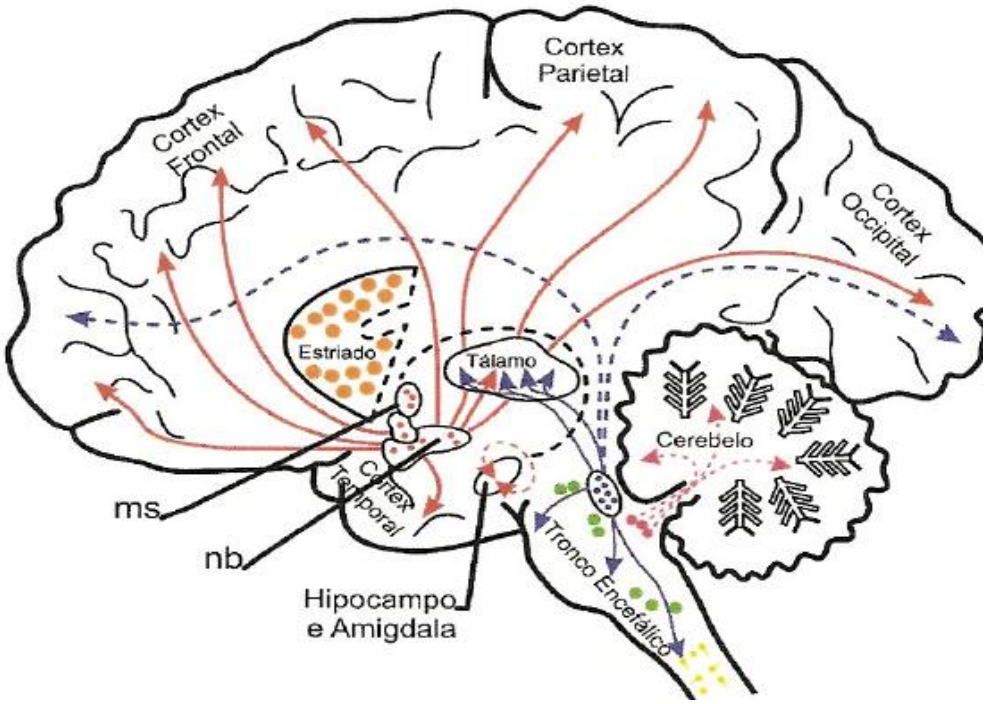
3.1.3 Cafeína e Sistema Colinérgico

Outro importante sistema modulatório é o sistema colinérgico. A ACh atua como um neuromodulador do SNC (para revisão ver Yakel, *in press*), tem um papel fundamental como neurotransmissor periférico excitatório, e contribui para a plasticidade sináptica em muitas áreas do cérebro (para revisão ver Yakel, *in press*) (Figura 5). A neurotransmissão colinérgica central predominante está relacionada à excitabilidade neuronal, alterações da liberação pré-sináptica de neurotransmissores, e disparo coordenado de neurônios (Kawai *et al*, 2007;. Arroz & Cragg, 2004; Zhang & Sulzer, 2004) e regulada pela enzima acetilcolinesterase (AchE) por meio da hidrólise de ACh (Silver, 1974).

As ações da acetilcolina ocorrem por meio de duas classes de receptores metabotrópicos: receptores muscarínicos (mACh) e nicotínicos (nACh) (para revisão ver Picciotto *et al*., 2000; Wess, 2003a). Os receptores muscarínicos estão localizados pré e pós sinapticamente em todo o sistema nervoso (para revisão ver Yakel, *in press*) e os receptores nicotínicos estão localizados em gânglios, placa motora e SNC (Changeux *et al*, 1998; Picciotto, *et al*., 2001). A ação modulatória da ACh está relacionada a liberação de glutamato, GABA, DA, ACh, noradrenalina e serotonina via ativação de receptores nicotínicos (McGehee, *et al*, 1995;. Wonnacott, 1997). Também sendo importante na sincronização da atividade neuronal (Bucher & Goaillard, 2011; Kawai *et*

al., 2007) mediando respostas pós sinápticas por meio de receptores nACh no hipocampo (Bell *et al*., 2011;. Gu & Yakel, 2011) e no córtex (Arroyo, *et al*., 2012).

Figura 5. Sistema colinérgico (encéfalo humano). Localizações dos principais grupos de corpos celulares e tratos de fibras colinérgicas são mostrados em vermelho.



(Fonte: Adaptado de PERRY *et al*. (1999), modificado por MAZZANTI, 2007)

Pedata e colaboradores (1984) demonstraram que uma dose de cafeína 50 µM produz aumento na liberação de acetilcolina (ACh) em fatias de córtex cerebral de ratos. Em 2005, Carter e colaboradores demostraram que a administração oral de cafeína foi capaz de aumentar os níveis extracelulares de ACh, por meio de um antagonismo seletivo dos receptores A_1 em hipocampo de ratos, sendo esta a estrutura envolvida com aprendizagem (Blozovski, 1985) , memória (Izquierdo *et al*., 2008) e cognição (Hurst *et*

al., 2012), onde melhor se detecta as relações entre sistema adenosinérgico e colinérgico (Murray *et al*., 1982).

Estudo realizado por Benington e colaboradores em 1995 demostrou que a administração de agonista adenosinérgico (N6-cyclopentyl adenosine –CPA) mimetizou os efeitos da privação de sono (Benington *et al*., 1995) e de sono não-REM (Schwierin *et al*., 1996). Já a administração de um antagonista seletivo mimetizou os efeitos da cafeína (O'Connor *et al*., 1991). O local em que a adenosina exerce estes efeitos de ligados ao sono não é conhecido, mas os neurônios colinérgicos da região mesopontina, que estão sob controle do receptor A_1, provavelmente sejam os responsáveis (Rainnie *et al*., 1994).

Sabe-se que a depuração de ACh aumenta após o uso de teofilina (Murray, *et al*., 1982) e que a cafeína pode afetar os níveis de acetilcolina e seu metabolismo cortical (Phillis *et al*., 1980;. Murray *et al*., 1982; Carter *et al*., 1995) em animais. Além disto, o aumento da atividade colinérgica observada após a administração de doses de cafeína, semelhantes às utilizadas por humanos, pode fornecer uma base neurobiológica para os efeitos psicoestimulantes da cafeína (Carter *et al*., 1995).

A adenosina inibe a liberação de neurotransmissores pré-sinápticos e deprime a excitabilidade neuronal pós-sináptica pela sua ação em receptores A_1 (Schubert *et al*., 1982,. Segal, 1982). Dessa maneira, a ação da adenosina em receptores A_1 pode induzir o sono, talvez em parte pela diminuição da atividade nos neurônios colinérgicos (Fredholm *et al*., 1999). Isto ocorre devido à inibição da atividade da adenilato ciclase, aumento do efluxo de potássio e redução do influxo sódio (Fredholm & Dunwiddie, 1988).

Considerando que a ação dos receptores A_1 está bem estabelecida, o papel dos receptores A_{2A} é controverso. Alguns estudos mostraram que os receptores A_{2A}

estimulam a liberação de ACh em sinaptossomas colinérgicos isolados a partir do estriado (Richardson *et al*., 1987; Brown *et al*., 1990; Kirk & Richardson 1994). Em estudos realizados por Cunha em 1994 e 1995, mostram que a estimulação de receptores A_{2A} em neurônios colinérgicos do hipocampo, aumentam a liberação de ACh (Cunha *et al*. 1994a, 1995), mas que os efeitos inibitórios mediados pelos receptores A_1 são mais facilmente detectáveis (Fredholm, 1990). Assim, liberação de ACh a partir destes neurônios parecem ser reguladas de forma oposta, por inibição de receptores A_1 ou por estimulação de receptores A_{2A} (Cunha *et al*., 1994b).

Dessa forma, destaca-se a importância de estudos que avaliem os efeitos do consumo gestacional de cafeína e suas consequências sobre o desenvolvimento fetal e neurotransmissão, tanto no que tange a avaliações farmacológicas, quanto as moleculares.

4 OBJETIVOS

4.1 Objetivo principais

Avaliar os efeitos da administração crônica de cafeína no período gestacional e neonatal em ratos sobre parâmetros comportamentais, neuroquímicos e moleculares.

4.2 Objetivos Secundários

Avaliar os efeitos da administração crônica de cafeína no período gestacional e neonatal de ratos a partir dos seguintes parâmetros:

a) Reflexos neurológicos da prole a partir do P1 até P14, utilizando como parâmetros o comportamento de endireitamento postural e resposta a geotáxia negativa;
b) Locomoção e atividade exploratória em campo aberto;
c) A atividade e a expressão gênica de acetilcolinesterase em hipocampo;
d) Limiar nociceptivo, utilizando aparelho de *tail-flick;*
e) Funcionalidade dos receptores de adenosina, por meio da administração de agonista e antagonista de receptores adenosinérgico do tipo A_1;
f) A atividade de hidrólise de nucleotídeos em sinaptossomas de medula espinhal.

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6 ARTIGOS CIENTÍFICOS

ARTIGO I: Gestacional caffeine intake alters neuromotor development and acetylcholinesterase activity in hippocampus of rat offspring.

Status: a ser submetido

GESTACIONAL CAFFEINE INTAKE ALTERS NEUROMOTOR DEVELOPMENT AND ACETYLCHOLINESTERASE ACTIVITY IN HIPPOCAMPUS OF RAT OFFSPRING.

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Abstract

Caffeine is the second most consumed substance at worldwide; it is quickly absorbed and can rapidly cross the placenta. A discreet intake of caffeine by the mother may have significant effects upon the fetus. The A_1 and A_{2A} receptors are susceptible to the action of caffeine; it can affect acetylcholine (ACh) levels and its metabolism in the brain, as well as, promotes an increase cortical of acetylcholinesterase (AChE) dose dependent. The objective was to evaluate the effect of chronic caffeine intake in gestational period and breastfeeding on behavioral response, such as righting reflex (RR), negative geotaxis (NG), open field test (OF). In addition, the AChE activity and its expression in hippocampus of 14 days rat offspring were evaluated. The mating of adult female Wistar rats was confirmed by sperm presence in vaginal smears. On the first day of pregnancy, rats were divided into three groups: (1) control, (2) caffeine and (3) washout caffeine. The treatment to dams was conducted during entirely gestation and lactation until pups achieve 14 days of age, the test were conducted between postnatal day 1 (P1) and P14 according to the protocol. The washout group the caffeine solution was changed to tap water until P7. ANOVA following by Tukey when necessary was used, and $P<0.05$ was considered significant difference. Caffeine treatment increased the latency in both neurological reflexes (RR and NG), and the washout caffeine group had a small time improvement when compared to the control. Caffeine decrease outer crossings and locomotion in relation to control and washout caffeine group increase inner crossings in relation to other groups, in OF. And decrease rearing only in relation to control. The caffeine group showed a decrease in the AChE activity without effect in the transcriptional regulation of AChE (RT-qPCR). In conclusion, we can suggest that caffeine promoted post-transcriptional or post-translational modulation effect in the AChE enzyme. In addition, behavioral alterations are promoted after chronic caffeine exposure. Further studies are necessary to clarify our results, such as ACh receptors expression.

Keywords: caffeine, gestation, adenosine, acetylcholine and neurological reflexes.

1 Introduction

Caffeine (1,3,7-trimethylxanthine) is considered the most widely consumed substance at worldwide, due to promote acute benefits for physiological, psychomotor and cognitive performance (Heckman et al., 2010; Einöther and Giesbrecht, 2013). It is the second most consumed substance after water; around 2.5 billion cups (30 mL / cup) of coffee per day worldwide (Heckman et al., 2010; Byungsung et al., 2012).

The consumption of caffeine is so broad that 98% of women of childbearing age consume caffeine regularly, with 72% of these are still making use during pregnancy (James, 1991). Caffeine has rapidly absorption and crosses the placenta reaches the fetus, which in turn has decreased metabolism and elimination due a lack of cytochrome P-450 activity, resulting in a prolonged half-life (Knutti et al. 1982; Kirkinen et al., 1983; Soellner et al., 2009). Thus, a discreet intake of caffeine by the mother may have significant effects upon the fetus (Serapiao-Moraes et al., *in press*).

It is interesting to highlight that caffeine can act at different systems of neurotransmission. One of caffeine's actions is well established as an adenosine antagonist (Ferre et al., 2008; Sawynok et al., 2010; Sawynok, 2011). The adenosinergic receptors more susceptible to its action are A_1 and A_{2A} receptors (Einöther and Giesbrecht, 2013). Adenosine is an endogenous normal cell constituent, its levels regulated by intracellular balance of various enzymes and has an important role in the nociceptive response (Sawynok, 1998). The interaction between caffeine and the neural development induced by adenosine during gestation and early post-natal (Zimmermann, 2006) needs to be more investigated.

In addition, the caffeine can affect acetylcholine (ACh) levels and its metabolism in the brain (Phillis et al, 1980; Murray et al, 1982, Katsura et al, 1991; Carter et al, 1995). The turnover of ACh is increased after the use of theophylline

(Murray et al., 1982). It is known that ACh is a key neurotransmitter in the brain involved in cortical activation, attention, memory, learning, pain, motor control tone, movement and control of autonomic functions (Herlenius and Lagercrantz, 2004).

The caffeine promotes an increase cortical of acetylcholine (ACh) dose dependent and an increase in the cholinergic activity in relevant doses of caffeine found in humans can provide a basis for the psycho-stimulant effect of caffeine (Carter et al., 1995). In addition, the cholinergic system is under development during gestation and early post-natal life periods (Gould et al., 1991; for review see Abreu-Villacxa et al., 2011).

Take into account a relationship between adenosine and ACh, the hippocampus appears to be the best structure to investigate the effects of adenosine upon ACh (Murray et al., 1982; Schulz et al., 2012). Some studies have shown that A_{2A} receptors stimulate the release of ACh in cholinergic synaptosomes isolated from the corpus striatum (Richardson et al., 1987; Brown et al., 1990; Kirk and Richardson, 1994). The action ACh itself, its co-transmitter ATP and ATP-hydrolyzation product adenosine were studied on quantal and non-quantal ACh (Galkin et al., 2001; Nikolsky et al., 2004; Silinsky, 2004; Veggetti et al., 2008; Burnstock, 2009; Voss, 2009).

The caffeine use during the pregnancy may trigger several biochemical processes during fetal brain development, which can to be involved in many chronic health problems and mental disorders like anxiety, learning, emotional and depression that can last a lifetime (Li et al., 2012). Thus, the objective of this study was to evaluate the effect of caffeine intake in gestational period and breastfeeding on neuromotor development, the AChE activity and its expression in hippocampus of 14 days rat offspring.

2 Materials and Methods

2.1 Animals

Adult female Wistar rats (weighing approximately 220g) of approximately 90 days of age were performed vaginal lavage to verify the estrous cycle. In their fertile phase females were housed at the evening with knowingly a male fertile. In subsequent morning, males were removed and the vaginal smears collected. Mating was confirmed by sperm presence in vaginal smears. All animals were housed individually in home cages made of Polypropylene material (49 x34x16cm) with laboratory-grade. All animals were maintained in a standard 12:12 light-dark cycle (lights on at 07:00 a.m. and lights off at 07:00 p.m.) in a controlled environment (22±2°C). Animals had *ad libitum* access to water and chow. All experiments and procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol No. 110034) and conformed to the Guide Laboratory for the care and use of animals (2011). Animal handling and all experiments were performed in accordance with international guidelines for animal welfare and measures were taken to minimize animal pain and discomfort. The experiment used the number of animals necessary to produce reliable scientific data.

2.2 Experimental design

On the first day of pregnancy, rats were divided into three groups according to the treatment they received: (1) control group, which received only tap water, (2) caffeine group, which received only a solution of 0.3 g/l caffeine diluted in tap water – the dose of caffeine given produced blood levels in dams comparable to those obtained in humans after consumption of ~ 3 cups of coffee (Björklund et al., 2008) and (3) washout group, which receives the same solution of 0.3 g/L caffeine diluted in tap water

until the seventh day after birth and were replaced with tap water. The treatment to dams was conducted during entirely gestation and lactation until pups achieve 14 days of age, and the behavior were evaluated from second to fourteenth day of postnatal life.

2.3 Chemicals

Caffeine, Trizma Base, ethylenedioxy-diethylene-dinitrilo-tetraacetic acid (EDTA), ethylene glycol bis(beta amino ethylether)-N,N,N0,N0-tetraacetic acid (EGTA), sodium citrate, Coomassie Blue G, bovine serum albumin, acetylthiocholine, and 5,50-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co (St. Louis, MO, USA). TRIzol1 reagent, Platinum1 Taq DNA Polymerase and SYBR1 Green I were purchased from Invitrogen (Carlsbad, CA, USA). ImProm-IITM Reverse Transcription System was purchased from Promega (Madison, USA). All other reagents used were of analytical grade.

2.4 Neuromotor development

2.4.1 Righting reflex

The pups were placed in a supine position and the latency time required to turn over their longitudinal axis to restore a normal prone position was measured. It was considered to be fully achieved when the pups turned 180° around their longitudinal axis, their four paws being in contact with the plane surface within the observed 120 s. Animals that did not turn over within 120 s were assigned this score. This test was carried out daily from P1 to P13.

2.4.2 Negative geotaxis

Negative geotaxis is a postural reaction bringing the animal in the upright position when it is placed downwards. The rat pups were placed on an inclined board (45°) 35 cm length with its head pointing down. The animals had to turn around and crawl up the slope for 180° in order to bring their snout upwards and climb up the board with their forelimbs reaching the upper rim. In cases the animals did not succeed at this task within the observed 120 s, the test was considered negative. The day of appearance of geotaxis and the time to reach the upper end of the board were recorded. The test was carried out daily from P4 to P13.

2.5 Open Field Test (OF)

The behavior assessment was rated the fourteenth day of postnatal life and was performed in a varnished wood cage, measuring 60 x 40 x 50 cm, with the inside lined with glass. The floor was recovered with linoleum divided into 12 rectangles of 13.0 x 13.0 cm with dark lines. The animal was gently placed in the left back corner, and left free to explore the surroundings for 5 min (Bianchin et al., 1993; Carlini et al., 2002). The number of crossings performed by each animal was taken as locomotor activity (Roesler et al., 1999). The latency to leave of the first quadrant was taken as an anxiety measure (Britton and Britton, 1981; Lister, 1990). Rearing was defined as the moment the rat rose up on its hind legs, ending when one or both front paws touched the floor again (Wells et al., 2009), and it was evaluated as exploratory activity (Silveira et al., 2005). Grooming was defined as licking/washing of head and body. Therefore, it was evaluated as a biological function of caring for the body surface (Spruijt et al., 1992). The start of a trial occurred immediately after the rat was placed in the environment for scoring purposes. In this test, the animal was recorded as entering a new area when all

four paws crossed the boundary into a different marked-out area. Five measures were taken during the 5-min test sessions: (1) number of line crossings (i.e. horizontal activity), outer and inner crossings in the OF; (2) latency to leave of the first quadrant; (3) grooming (time in seconds); (4) number of rearing behaviors (i.e. vertical activity). The box was cleaned in between each trial.

2.6 Sample collection

Pups were killed by decapitation at 14 day of age, and the brain structures were separated on a cold surface, and the samples were immediately frozen according to the experiment.

2.7 Protein determination

Protein was measured by the Comassie Blue method (Bradford, 1976) using bovine serum albumin as standard.

2.8 Determination of AChE activity

The hippocampus was homogenized on ice in 60 volumes (v/w) of Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) using a Potter–Elvehjen-type glass homogenizer. The rate of hydrolysis of acetylthiocholine iodide (0.88 mM) was determined in a final volume of 300 μL, with 33 μL of 100 mM phosphate buffer, pH 7.5 mixed to 2.0 mM 5,5′-dithionitrobis 2-nitrobenzoic acid (DTNB). Samples containing 5 μg protein and the reaction medium specified above were preincubated for 10 min at 25 °C. The hydrolysis of acetylthiocholine iodide was monitored in a microplate reader by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) (Ellman et al., 1961). Controls without the

homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of the substrate. AChE activity was expressed as μmol thiocholine (SCh). h^{-1}. mg $protein^{-1}$. All experiments were performed in quadruplicate.

2.9 Gene expression analysis by quantitative real time RT-PCR (RT-qPCR)

Gene expression analysis was carried out only when kinetic alteration occurred. Total RNA was isolated with Trizol® reagent (Invitrogen, Carlsbad, California, USA) in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 μg of total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 μL using 12.5 μL of diluted cDNA (1:50 for *Hprt1*, *Rlp13α* and *ache*), containing a final concentration of 5 M betaine (Sigma-Aldrich), 0.2 x SYBR® Green I (Invitrogen), 100 μM dNTP, 1 x PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primers. The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95°C, 40 cycles of 15 s at 95ºC for denaturation, 35 s at 60 °C for annealing and 15 s at 72°C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl) and the stability of the references genes, *Hprt1* and *Rlp13α* (*M-value)* and the optimal number of reference genes according to the pairwise variation

(*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

3 Statistical Analyses

Data were expressed as mean ± standard error of the mean (S.E.M.). It was used ANOVA repeated measures followed by Bonferroni test (for time) and ANOVA followed by Student-Newman-Keuls test - SNK (for group) to righting reflex and negative geotaxis. One-way ANOVA following by Tukey when necessary was used for different analysis. Differences were considered statistically significant if $P<0.05$.

4 Results

4.1 Neuromotor development

4.1.1 Righting reflex

The caffeine and washout caffeine groups presented an increase in the latency to the righting reflex in relation to the control group (Figure 1).

Insert about here Figure 1

4.1.2 Negative geotaxis

Prenatal caffeine exposure significantly affected the number of subjects that exhibited the negative geotaxis reflex (Figure 2). Significant differences were observed at the mean during the following days of testing, the caffeine and washout caffeine

group was significantly lower than the control group and the washout group had a small time improvement compared to the control group (Figure 2).

Insert about here Figure 2

4.2 Open Field Test (OF)

At P14, there were no differences among the groups in the latency to leave the first quadrant out (one-way ANOVA/Tukey, $F_{(2,46)}$= 1.60, P>0.05; Figure 3a). There was a significant decrease in outer crossing and locomotion in the caffeine group when compared to control group (one-way ANOVA/Tukey, $F_{(2,46)}$= 5.37, $F_{(2,46)}$= 4.85, respectively, P<0.05; Figure 3b and 3f). The washout group was not different in outer crossing and locomotion in comparison to caffeine and control groups (one-way ANOVA, P>0.05). There was a significant increase in inner crossing in the washout group when compared to control and caffeine groups (one-way ANOVA/Tukey, $F_{(2,46)}$=5.57, P<0.05; Figure 3c). The washout group showed a decrease in number of rearing in comparison to control group (one-way ANOVA/Tukey, $F_{(2,46)}$= 3.32, P<0.05; Figure 3d). The caffeine group was not different from the other groups (one-way ANOVA, P>0.05).

Insert about here Figure 3

4.3 Determination of AChE activity

Caffeine group presented a significant decrease in AChE activity at P14 when compared to the control group (control: 39.20±1.16 mmol SCh h^{-1} mg $protein^{-1}$; caffeine: 17.39 ±8.40 mmol SCh h^{-1} mg $protein^{-1}$; one-way ANOVA/Tukey; $F_{(2,9)}$=4.51, P<0.05; Figure 4), which corresponds to approximately 55.62%. The down-regulation of AChE activity after exposure to 0.3 g/L could be a consequence of transcriptional control. The washout group showed no difference from other groups (30.28 ± 2.84 mmol SCh h^{-1} mg $protein^{-1}$, one-way ANOVA, P>0.05; Figure 4).

Insert about here Figure 4

4.4 Gene expression analysis by quantitative real time RT-PCR (RT-qPCR)

In order to determine if transcriptional regulation of AChE has occurred, a RT-qPCR analysis was carried out. The results have shown that *ache* transcript levels in the 0.3 g/L group were not altered when compared to the control group (one-way ANOVA, P>0.05; Figure 5) suggesting that the down-regulation of brain AChE is not directly related with the transcriptional control.

Insert about here Figure 5

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5 Discussion

In the present study we showed that chronic caffeine intake during gestational and breast-feeding was able to promote an increase in the latency of righting reflex and negative geotaxis behavior, together with decrease in the outer crossing and total

locomotion in the open field test. The washout group was able to increase inner crossing and decrease rearing, with caffeine group presenting a partial effect in rearing behavior. In addition, the chronic caffeine exposure decreased the AChE activity in relation to control group.

In our study we observed an average delay of reflexes in rats offspring in the caffeine group showing a slow performance on reflexive tests, demonstrared damages in the attached reflexes to spatial evaluation and motor injury. The daily observations of reflexes (postural reflex and negative geotaxis) are sensitive indicators of early stages of development of the newborn (Martin et al., 1976; Gibb and Kolb, 2005). The gray matter of the spinal cord is an area of integration to the spinal cord reflexes and other motor functions, and the negative geotaxis is usually analyzed in young mice and appears to be a form of compensatory response or even an emergency response to postural instability in an inclined surface (Motz and Alberts, 2005).

The prenatal caffeine exposure decreased the exploratory activity and the locomotion. Caffeine induces sensitization in the locomotor activity (Meliska et al., 1985; Cauli et al., 2003; Simola et al., 2006; Hsu et al., 2010) showing that low doses of caffeine ingested by rats, amounts consumed by light human users (~3 cup of coffee/day) induce a form of behavioral plasticity (Somani and Gupta, 1988; Reagan-Shaw et al., 2007; Ball and Poplawsky, 2011). It is know that acetylcholine (ACh) is a key neurotransmitter in the brain involved in cortical activation, attention, memory, learning, pain, motor control tone, movement and control of autonomic functions (Herlenius and Lagercrantz, 2004). The caffeine half-life is increased during the neonatal period (Aranda et al., 1979) due the relative immaturity of some demethylation and acetylation pathways (Aranda et al., 1974; Carrier et al., 1988), thus it is possible that caffeine accumulation may be triggering several biochemical processes during fetal

brain development, which when affected can cause many chronic health problems and mental disorders like anxiety, learning, emotional and depression that can last a lifetime (Li et al., 2012).

Studies with electrophysiological have shown that the hippocampus is particularly sensitive to the inhibitory effects of adenosine and its analogues (Dunwiddie et al., 1981; Schulz et al., 2012). Indeed, the density of A1 receptors is higher in the hippocampus than in any other area of the brain (Murphy and Snyder, 1982; Sasaki et al., 2011). Other important point is the effects of adenosine on ACh turnover are greatest in the hippocampus (Murray et at, 1982). In vitro experiments demonstrate that ACh release is depressed at the level of the cholinergic nerve terminal by the action of endogenous adenosine at A1 receptors (Jackisch et al., 1984).

We found a decrease in the acetylcholinesterase (AChE) activity in caffeine exposure animals. It is possible that this result can be associated with antagonism of adenosine receptors sensitive to caffeine, since the cholinergic transmission is regulated by adenosine (Haubrich et al., 1981; Forloni et al., 1986). In addition, the RT-qPCR analysis demonstrated that caffeine exposure did not alter *ache* mRNA levels in rat hippocampus. Therefore, we can conclude that the process that inhibits AChE is probably involves a post-transcriptional or post-translational modulation of this enzymatic activity (Brito et al., 2012). Since that adenosine A1 receptors have been shown to be down regulated by adenosine analogs via a post-translational mechanism (Ramkumar et al., 1991). After chronic treatment with caffeine, it was observed a down regulation of adenosine receptors in neonatal life and pregnancy (Léon et al., 2002, da Silva et al., 2003).

In summary, our findings show that moderate doses of caffeine can induce a delay in reflexive tests, by promoting reflexes losses related to spatial evaluation and

motor injury, cognitive performance, behavioral alterations, and changes in acetylcholinesterase activity. Given the widespread use of caffeine, research involving the effects of repeated exposure to caffeine during gestational period and early postnatal life upon behavioral, biochemical and molecular levels needs to be developed.

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Legends:

Figure 1. Effect of prenatal caffeine exposure on postural righting reflex. Bars represent the mean±S.E.M. (n=8-16). The asterisk (*) indicates a significant difference compared to control group (ANOVA/SNK, $F_{(2,40)} = 2.43$, $P<0.05$).

Figure 2. Effect of prenatal caffeine exposure on negative geotaxis response. Bars represent the mean±S.E.M. (n=8-16). The asterisk (*) indicates a significant difference compared to control group (ANOVA/SNK, $F_{(2,40)} = 0.69$, $P<0.05$).

Figure 3. Effects of oral caffeine on (a) latency to leave the first quadrant, (b) outer crossings, (c) inner crossings, (d) rearing, (e) grooming and (f) locomotion behaviors in the open field test. Bars represent the mean±S.E.M. (n= 10-22). *significant difference from control group and $^{\#}$significant difference from caffeine and control groups (one-way ANOVA/Tukey, $P<0.05$).

Figure 4. AChE activity in hippocampus of rat offspring. Bars represent the mean ±S.E.M. (n = 4). The specific enzyme activity is reported as mmol SCh $h^{-}1$ mg $protein^{-}$ 1. *significant difference from control group (one-way ANOVA/Tukey; $P< 0.05$).

Figure 5. RT-qPCR analysis. Relative AChE expression profile after caffeine exposure (0.3 g/L) on hippocampus of rat offspring. Bars represent the mean ±_ S.E.M. (n = 4). No differences found (one-way ANOVA/Tukey; $P> 0.05$).

Figures:

Figure 1

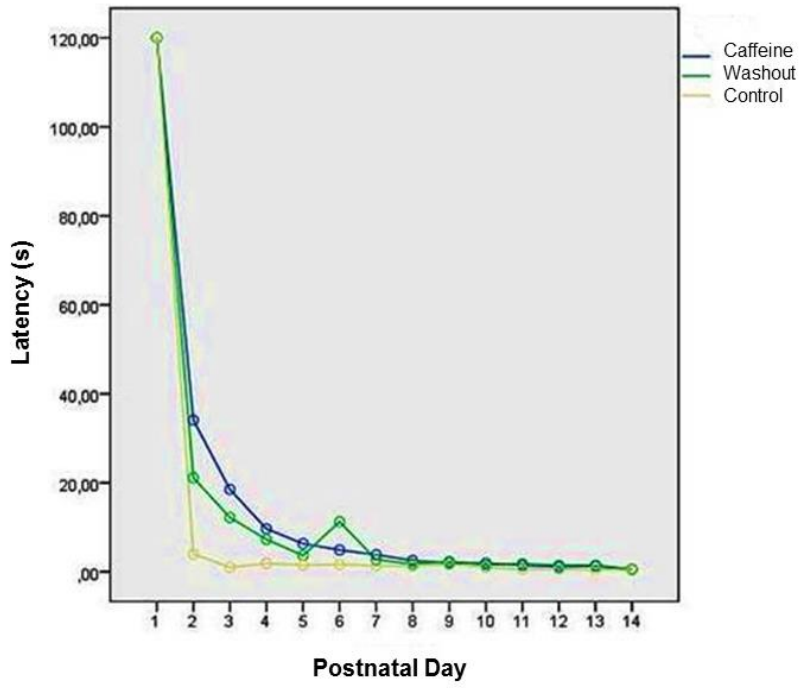


Figure 2

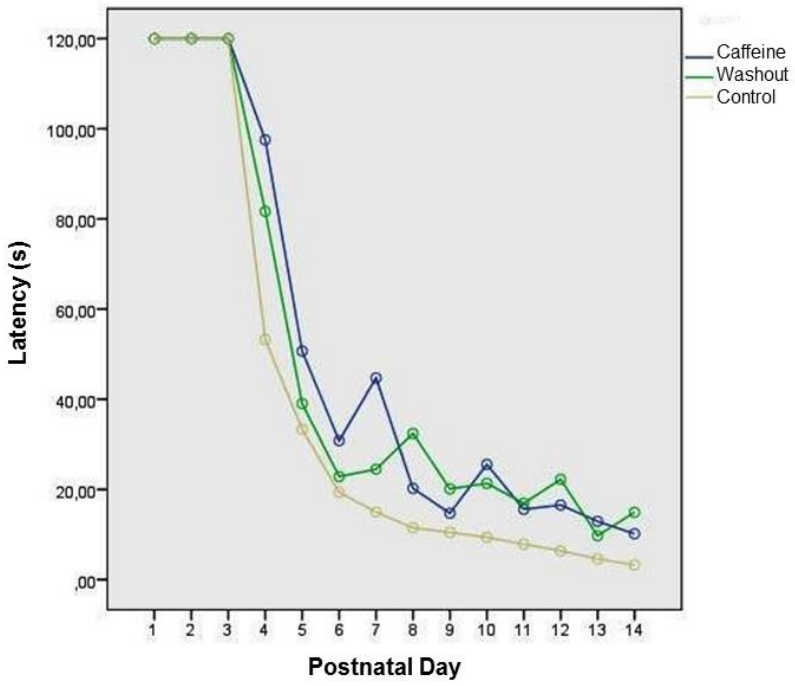


Figure 3

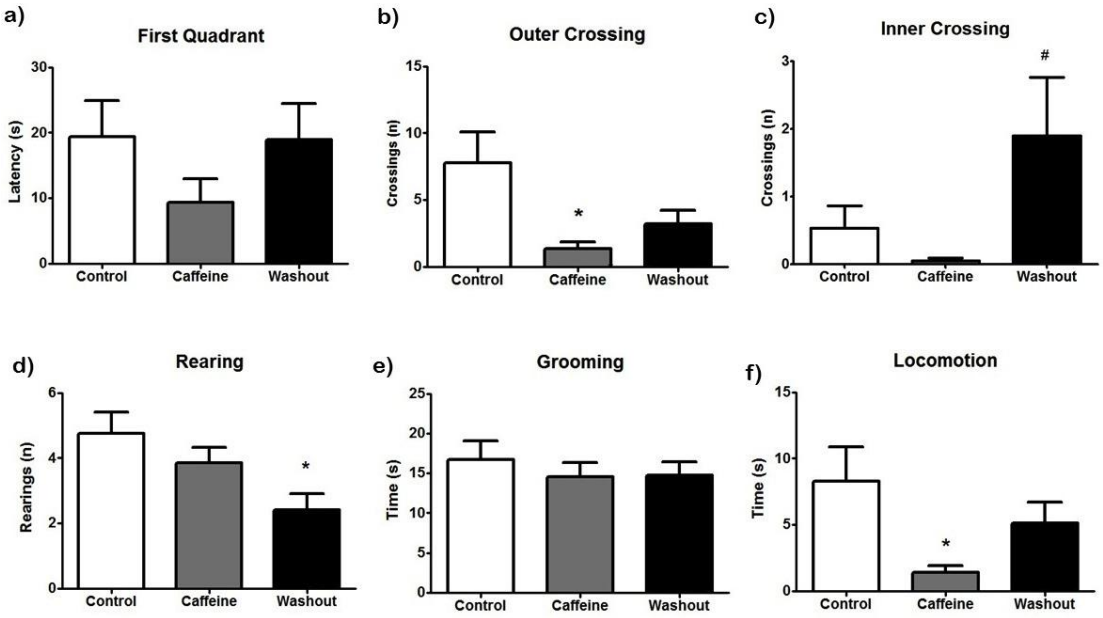


Figure 4

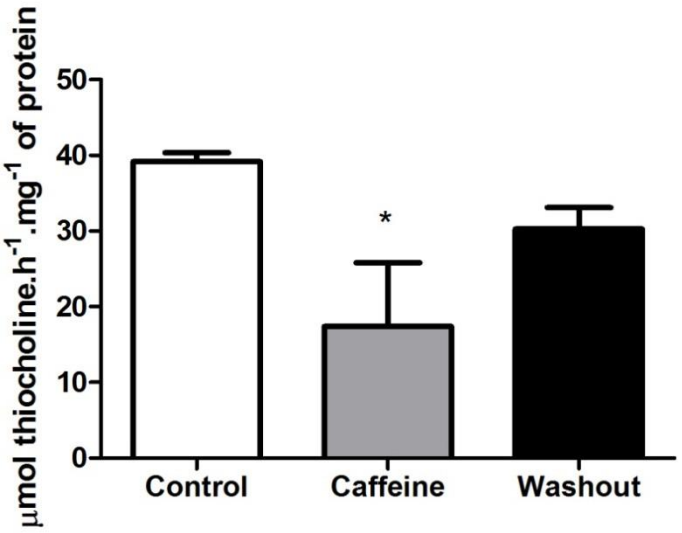
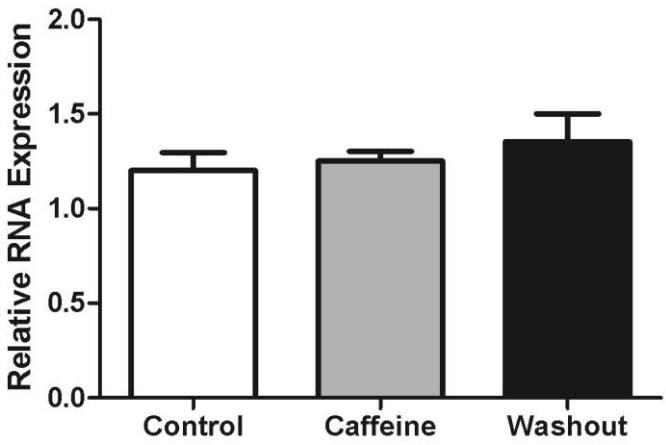


Figure 5


Relative RNA Expression
2.0
1.5
1.0
0.5
0.0
Control
Caffeine
Washout

ARTIGO II: Chronic caffeine exposure alters the adenosinergic agonist response in rats.

Status: a ser submetido

CHRONIC CAFFEINE EXPOSURE ALTERS THE ADENOSINERGIC AGONIST RESPONSE IN RATS.

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Conflict of Interest: There was no financial relationship between any of the authors or any commercial interest in the outcome of this study.

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Abstract

Caffeine is a common substance found in a wide variety of beverages and foods. It is extensively consumed as a psychostimulant drug. The chronic exposure during gestation and breastfeeding may be involved in behavioral and biochemical alterations. Caffeine acts blocking A_1 and A_{2A} adenosine receptors. The A_1-subtype is associated with a modulatory effect on pain transmission at the spinal cord level. Thus, we investigated the effect of administration of agonists and antagonists of adenosine A_1 receptor in rats submitted to chronic caffeine during gestation and breastfeeding. In addition we evaluated the NTPDase and 5'-nucleotidase activities and nociceptive behavior (tail flick latency). The mating of adult female Wistar rats was confirmed by sperm presence in vaginal smears. On the first day of pregnancy, rats were divided into three groups: (1) control, (2) caffeine and (3) washout caffeine. The treatment to dams was conducted during entirely gestation and lactation until pups achieve 14 days (P14). The washout group the caffeine solution was changed to tap water until P7. The tests were conducted at P14. ANOVA following by Tukey when necessary was used, and $P<0.05$ was considered significant difference. At tail flick test, there was no difference between the groups at baseline. When we used N6 Cyclopentyladenosine (CPA) to evaluate the functionality of adenosine A1 receptors caffeine group presented a decreased response and the washout showed this effect partially reverted, demonstrating that is not a long lasting effect. However, there were no differences in NTPDase and 5'-nucleotidase activities and with 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX) administration. Our results demonstrate the importance to extending studies related to chronic caffeine administration in gestational and breast-feeding in formation and maturation periods of CNS. Further studies are necessary to elucidate the real mechanisms involved in this pharmacological alterations found.

Keywords: caffeine, pregnancy, A_1 receptors, tail flick latency, ectonucleotidases.

1 Introduction

Caffeine is a substance found in a wide variety of beverages and foods (Einöther and Giesbrecht, 2013). It is characterized as a methylxanthine and is able to stimulate the central nervous system (CNS) making caffeine the most used psychoactive worldwide (Ferre, 2008). Around 80% of the world's population consumes caffeinated products every day (Heckman et al. 2010) including women of childbearing age (James, 2001).

Caffeine is a hydrophobic compound, readily crosses biological membranes including the placenta (Kirkinen et al., 1983). Due to the inability of the fetus metabolize caffeine; the half-life is increased during gestation (Fredholm, 1995; Bakker et al., 2010). The principal caffeine metabolism enzyme, cytochrome CYP1A2, is absent in the placenta and fetus (Aldridge and Aranda, 1979).

Additionally, it is important to highlight the use of caffeine in pharmaceutical drugs. Among the therapeutic uses of caffeine stands its use as treatment of apnea in premature neonates (Schmidt et al., 2006) and for treating headache after spinal anesthesia (for review see Tavares and Sacata, 2012). Furthermore, it is commonly associated with analgesic and anti-inflammatory drugs particularly for the treatment of headache (Derry et al., 2012).

The caffeine acts blocking A_1 and A_{2A} adenosine receptors (Fredholm, 1980; Huang et al, 2011), yielding increased levels of cyclic AMP by phosphodiesterases inhibition (Soyka, 1979).

The adenosine (ADO) is a CNS modulator (for review see Sawynok, 2011) acting through G protein-coupled receptors type A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 2001). It has been proposed that endogenous adenosine formation is involved in physiological pain control at the spinal cord level, and its release appears to have

functional importance with respect to regulating nociceptive thresholds (Jurna, 1984, Sawynok et al., 1986) and mediating a component of spinal analgesia by morphine and 5-HT (Delander and Hopkins, 1986; 1987).

Animal studies have demonstrated adenosine- and adenosine analog-mediated inhibitory influences on presumed nociceptive reflex responses (For review see Sollevi, 1997; Sawynok, 1998). The adenosine A1 receptors are associated with a modulatory effect on pain transmission at the spinal cord level (For review Keil and Delander, 1996). These agonists' types appear to act presynaptically reducing neuronal excitability (Hass and Greene, 1988; Lambert and Teyler, 1991). Adenosine analogs have antinociceptive properties in a wide range of test systems, including that neuropathic pain, where pain-signaling mechanisms have been altered (Sawynok, 1998). The endogenous ligand adenosine is clinically available and has been tested as an analgesic by intrathecal (i.t.) administration (Von Heijne et al., 1999).

The adenosine enzymatic production is involved a cascade of ectonucleotidases (E-NTPDases and 5'nucleotidase enzymes; for review see Kowaluk and Jarvis, 2001). The E-NTPDases control the availability of ligands for both nucleotide and nucleoside receptors and the duration of receptor activation. These enzymes may provide a protective function by maintaining extracellular ATP/ADP and adenosine within physiological concentrations (for review see Burnstock, 2013). ATP as well as other nucleotide agonists for P_2-purinoceptors could be degraded by ectonucleotidases. The hydrolysis of nucleotides is related to a large number of physiological processes.

Considering the close relationship between caffeine and adenosine, and their role in the pain response, in this study we investigated the effect of administration of agonists and antagonists of adenosine A_1 receptor in rats submitted to chronic caffeine

intake during pregnancy and breastfeeding. In addition we evaluated the NTPDase and 5'-nucleotidase activities and nociceptive behavior in rats at P14.

2 Materials and Methods

2.1 Animals

Adult female Wistar rats (weighing approximately 220g) of approximately 90 days of age were performed vaginal lavage to verify the estrous cycle. In their fertile phase females were housed at the evening with knowingly a male fertile. In subsequent morning, males were removed and the vaginal smears collected. Mating was confirmed by sperm presence in vaginal smears. All animals were housed individually in home cages made of Polypropylene material (49 x34x16cm) with laboratory-grade. All animals were maintained in a standard 12:12 light-dark cycle (lights on at 07:00 a.m. and lights off at 07:00 p.m.) in a controlled environment (22±2°C). Animals had *ad libitum* access to water and chow. All experiments and procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol No. 110034) and conformed to the Guide Laboratory for the care and use of animals (2011). Animal handling and all experiments were performed in accordance with international guidelines for animal welfare and measures were taken to minimize animal pain and discomfort. The experiment used the number of animals necessary to produce reliable scientific data.

2.2 Experimental design

On the first day of pregnancy, rats were divided into three groups according to the treatment they received: (1) control group, which received only tap water; (2)

caffeine group, which received only a solution of 0.3 g/L caffeine diluted in tap water – the dose of caffeine given produced blood levels in dams comparable to those obtained in humans after consumption of ~ 3 cups of coffee (Björklund et al., 2008); and (3) washout group, which receives the same solution of 0.3 g/L caffeine diluted in tap water until the seventh day after birth (P7) and after replaced by tap water. The treatment to dams was conducted during entirely gestation and lactation until pups achieve 14 days of age (P14). The tests were conducted at P14.

2.3 Chemicals

Caffeine, Dimethyl sulfoxide (DMSO), NaCl 0.9%, N6 Cyclopentyladenosine (CPA) and 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX) (Sigma-Aldrich, USA). All other reagents used were of analytical grade.

2.4 Tail-flick measurement

Rats were tested for antinociception using the tail-flick test (TFL; Castilho et al., 2002). Each animal was placed on the apparatus and its tail was laid across a nichrome wire coil that was then heated using an electric current. The equipment was calibrated to obtain three consecutive baseline tail-flick latencies between 3 s and 5 s. If at any time the animal failed to flick its tail before the temperature reached 75 ◦C, the tail was removed from the coil to prevent damage to the skin. Three TFL baselines were taken at 3 min intervals. The animals were exposed to the tail-flick apparatus to acclimate to the procedure 24 h prior to the test session because the novelty of the apparatus can itself induce antinociception (Netto et al., 1987).

2.5 Drugs administration

The drugs used were N6-Cyclopentyladenosine (CPA, 3.35 mg/Kg) and 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.8 mg/Kg). CPA and DPCPX were dissolved in 0.9% NaCl and 5% dymethyl sulfoxide. All drugs were administered in a volume of 1.0 mL/Kg (i.p). Tail-flick latencies were measured before (basal measure), at immediately, 15, 30 and 60 min after the drug injection.

2.6 Synaptosomal preparation

The animals were killed by decapitation and the spinal cord was rapidly removed and gently homogenized in 10 vol of ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA and 5 mM HEPES, pH 7.5, with a motor driven Teflon-glass homogenizer. The synaptosomes were isolated as described previously (Nagy et al., 1984). Briefly, 0.5 ml of the crude mitochondrial fraction was mixed with 4 ml 8.5% Percoll solution and layered onto an isosmotic Percoll/sucrose discontinuous gradient (10%/20%). The synaptosomes that banded at the 10%/20% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The material was prepared fresh daily and maintained at 0–4°C throughout preparation.

2.7 Ectonucleotidases activity

The reaction medium used to assay ATPase–ADPase activities was described previously (Battastini et al., 1991). The medium contained (final concentration): 5 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 ml. The synaptosomal fraction (20 μL) was added to the reaction mixture and preincubated for 10 min at 37°C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1 mM

and was stopped by the addition of 200 μL 10% trichloroacetic acid. The samples were chilled on ice for 10 min and 100 μL samples were taken for the assay of released inorganic phosphate (Pi) (Chan et al., 1986). The reaction medium used to assay the AMP hydrolysis contained (final concentration): 1.0 mM $MgCl_2$, 100 mM Tris–HCl, pH 7.0 and 150 mM sucrose in a final volume of 200 μL (Heymann et al., 1984). The synaptosomal fraction (20 μL) was preincubated for 10 min at 37°C. The reaction was initiated by the addition of AMP to a final concentration of 1.0 mM and stopped by the addition of 200 μL of 10% trichloroacetic acid; 100 μL of samples were taken for the assay of released inorganic phosphate (Pi) (Chan et al., 1986). Incubation times and protein concentration were chosen in pilot studies to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct nonenzymatic hydrolysis of the substrates. All samples were run in duplicate. Protein was measured by the Coomassie Blue method (Bradford, 1976), using bovine serum albumin as standard. The specific activities were expressed as nmol Pi/min/mg protein.

3 Statistical analysis

Data were expressed as means ± standard error of the mean (S.E.M.). The two-way ANOVA repeated measure or one-way ANOVA following by Tukey when necessary were used according to the protocol experiment. Differences were considered statistically significant if $P<0.05$.

4 Results

4.1 Effect of chronic caffeine intake on drugs active at A_1 adenosine receptor

The caffeine group presented a decreased response after the use of CPA and the washout showed this effect partially reverted, demonstrating that is not a long lasting effect. We observed group effect, the caffeine group showed a decrease in the latency in relation to control group, however the washout group did not show any difference from the other groups (two-way ANOVA repeated measures, $F_{(2,60)}$= 5.476, P<0.05). It was observed drug effect; CPA administration was able to increase the latency in relation to DPCPX and vehicle groups (two-way ANOVA repeated measures, $F_{(2,60)}$=8.362, P<0.05). In addition, an interaction between time and drug was found (two-way ANOVA repeated measures, $F_{(2,53)}$=4.058, P<0.05; Figure 1).

Insert about here Table 1

4.2 Effect of chronic caffeine intake on ectonucleotidases activity in spinal cord

At P14, we did not observe any significant difference between groups in NTPDases and 5'-nucleotidase activities (**ATP** Control = 111.53 ± 24.42; Caffeine = 92.76 ± 19.87; Washout (104.72 ± 15.42); $F_{(2,16)}$= 0.23; **ADP** Control = 35,18 ± 6,78; Caffeine = 36,55 ± 6,95; Washout = 36,79 ± 3,85; $F_{(2,16)}$= 0.02; **AMP** Control = 3,81 ± 1,55; Caffeine = 3,15 ± 1,08; Washout = 3,48 ± 0.64; $F_{(2,16)}$ = 0.09; one-way ANOVA, P>0.05, n= 4-6; Figure 2).

Insert about here Figure 2

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5 Discussion

In the present study, the caffeine group displaying decrease CPA effects on nociception compared to the control group, despite no difference in threshold pain between the groups assessed by tail-flick test. The washout group showed less pronounced effect, but it was not different from others. We observed that CPA (adenosinergic agonist) administration promoted an increased in the latency in comparison to DPCPX and vehicle on control group. And interaction between time and drug was observed. In addition, no differences were observed between groups in NTPDases 5'nucleotidase activities in synaptosomes of spinal cord.

The nociception assessed by TFL is related to the reflex of the spinal cord (Irwin et al., 1951; Sinclair et al., 1988), but remains under control of supraspinal structures (Mitchell & Hellon, 1977). This test involves phasic pain, short duration of the noxious stimulus and a stimulation of minimal surface areas, and the pain threshold is measured. The test involves thermal stimulation of Aδ fibers (for review see Le Bars et al., 2001), and it is known that these fibers are present at birth (P0). It is suggested that possible changes may have occurred in the type Aδ fibers, for example a possible down-regulation after chronic caffeine administration. In the first three weeks of life there is an increase of C-type fibers in the spinal cord and the corresponding decrease in Aδ fiber type does not occur immediately. Therefore, the first three weeks of life, both fibers occupy the same space within the spinal cord (Fitzgerald, 1995). We suggest that the chronic caffeine administration at a stage of maturation can be promoting an alteration in the nociceptive response, resulting from an imbalance of nociceptive fibers.

Spinal adenosine A1 receptors are implicates in various pain models of antinociception (Dickenson et al., 2000; Sawynok, 1998). It is know that DPCPX mimicked the action of caffeine when given systemically (Sawynok and Reid, 2011).

In addition, it is important to note that the mRNA A_1 expression receptor in brain was first detected on gestation day 14 (G_{14}) and at gestational age 17 (G_{17}), the patterns of A_1 receptor expression in the brain were similar to the adults (for review see Burnstock, 2007). Additionally, a study revealed that was a decreased in adenosine A1 receptors after chronic caffeine exposure during gestation (80%), lactation (76%) and gestation plus lactation (80%) in male neonates, without variation in the level of mRNA coding adenosine A1 receptor (Lorenzo et al., 2010).

The chronic exposure to caffeine during gestational period, or breast-feeding period, can promote alterations in the function of this receptor. As shown in a study was observed down regulation in fetal brain after caffeine or theophylline treatment in pregnant rats (Léon et al., 2002). Mice pups chronically exposed in utero to caffeine presented appeared earlier and reached higher adult levels of adenosine receptors in the brains (most notably in the cerebellum) (for review see Burnstock, 2007). The exposure to caffeine during postnatal period demonstrated a transient increase (only at postnatal day 6) in the number of immunopositive neurons in brainstem areas, e.g. the caffeine modulates the ontogeny of the adenosine A1 receptor (Gaytan et al., 2006).

The first two weeks after birth is a critical development and synaptogenesis period for rats, because of the stimulation of the outgrowth and maturation of neurons (Hindley et al., 1997; Kalb and Agostini, 1993; Roskams et al., 1994) Therefore, the effect of caffeine on neonates may be in part caused by a blockade in adenosine A1 receptor or an indirect effect via dopaminergic (DA) interaction (Zylka, 2011). Other mechanisms of caffeine action are the inhibition of phosphodiesterase and mobilization of intracellular calcium, but these effects require higher concentrations of caffeine (for review see Lorist and Tops, 2003).

Multiple spinal systems, including opioid and adenosine purinergic systems

modulate nociceptive neurotransmission in the dorsal horn (Yaksh and Malmberg, 1994). Previous studies have demonstrated that manipulation of endogenous adenosine levels induces antinociception in the mouse tail-flick (Keil and Delander, 1992, 1994). Adenosine analogs have antinociceptive properties in experimental and clinical situations (Sawynok, 1998). Studies suggest that adenosine A1 receptor have influences on presumed nociceptive reflex responses (Keil and DeLander, 1996). In our study, we demonstrated the analgesic effect of CPA (A1 adenosinergic agonist) administered intraperitoneally (i.p.) pathway in control rats with 14 days old. In addition, an antagonist's adenosine receptors (i.t.) administration induces thermal hyperalgesia (Jurna, 1984, Sawynok et al., 1986). In behavioral studies, methylxanthines administered (i.t.) inhibit morphine(i.t.)-induced antinociception (Delander and Hopkins, 1986; 1987). These results indicated that an endogenous purinergic system might be active at spinal sites modulating nociception neurotransmission. Facilitation of this system would be expected to induced antinociception, whereas its inhibition would result in facilitated nociceptive neurotransmission.

In conclusion, our results demonstrate the importance to extending studies related to chronic caffeine administration in gestational and breast-feeding in formation and maturation periods of CNS, where different systems are being developed or matured. In the present study, we observed at P14 alterations in the nociceptive response, without neurochemical alterations of ectonucleotidases activities or in the nociceptive response of rats exposure to chronic caffeine administration in gestational and breast-feeding. Further studies are necessary to elucidate the real mechanisms involved in this pharmacological alterations found.

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Legends

Table 1.Effect of chronic caffeine intake on drugs active at A1 adenosine receptor. Data as mean±SEM. (two-way ANOVA repeated measure/Tukey; n= 13-28). *significant difference in comparison to control group (P<0.05). # significant difference in comparison to vehicle and DPCPX groups (P<0.05).

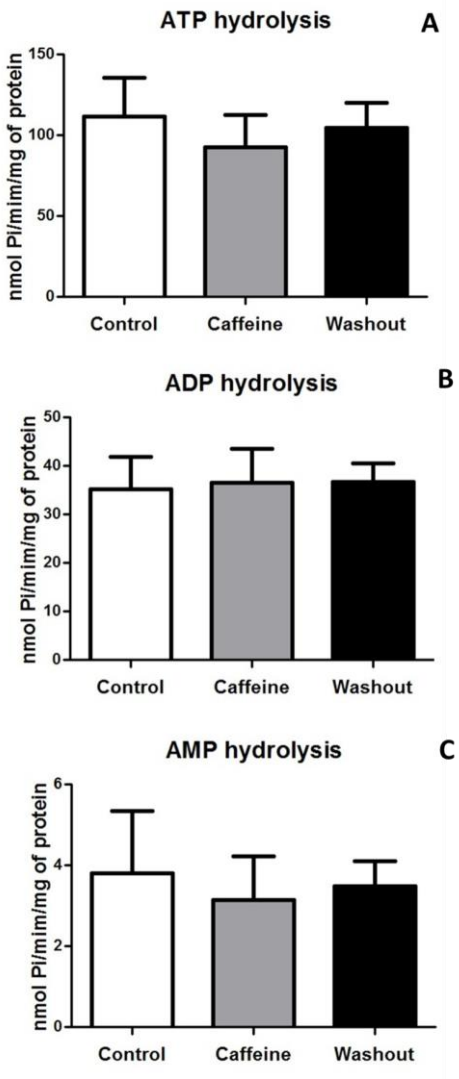
Figure 1. Effect of chronic caffeine intake on enzymatic activity of ectonucleotidases in spinal cord. Bars represent the mean ± S.E.M. No differences found (one-way ANOVA, P>0.05; n = 4-6).

Table and Figure

Table 1.

Group	Drug	Baseline (s)	Immediately (s)	15 min (s)	30 min (s)	60 min (s)
Control	Vehicle	4.78 ± 0.31	5.15 ± 0.29	5.05 ± 0.30	5.19 ± 0.43	4.97 ± 0.47
	CPA #	4.30 ± 0.24	5.73 ± 0.58	6.34 ± 0.54	6.52 ± 0.45	5.36 ± 0.32
	DPCPX	4.81 ± 0.45	4.74 ± 0.28	4.68 ± 0.39	4.78 ± 0.25	4.55 ± 0.46
Caffeine *	Vehicle	3.93 ± 0.26	3.80 ± 0.31	3.95 ± 0.41	4.55 ± 0.28	4.09 ± 0.18
	CPA #	4.30 ± 0.25	4.45 ± 0.21	5.76 ± 0.37	5.16 ± 0.48	4.66 ± 0.42
	DPCPX	4.88 ± 0.33	4.33 ± 0.29	4.44 ± 0.38	5.03 ± 0.26	4.57 ± 0.37
Washout	Vehicle	5.27 ± 0.12	4.95 ± 0.45	4.77 ± 0.82	4.50 ± 0.70	5.03 ± 0.35
	CPA #	4.85 ± 0.39	5.66 ± 0.59	6.06 ± 0.27	5.83 ± 0.24	5.28 ± 0.68
	DPCPX	4.64 ± 0.34	4.36 ± 0.39	3.52 ± 0.43	4.28 ± 0.47	5.06 ± 0.66

Figure 1.



7 CONSIDERAÇÕES GERAIS

Com os resultados obtidos nesta dissertação, podemos concluir que o tratamento materno crônico gestacional e pós-natal com cafeína pode causar na prole as seguintes alterações:

- ✓ Atraso nos tempos de realização das tarefas de reflexos neurológicos avaliados por endireitamento postural e resposta a geotaxia negativa, sendo que o grupo abstido de cafeína demonstrou um atraso menos pronunciado;
- ✓ Diminuição dos cruzamentos externos e do tempo total de locomoção, e o grupo abstido apresentou um aumento significativo no número de cruzamentos internos e diminuição do rearing no teste de campo aberto;
- ✓ Diminuição na atividade enzimática de AChE, mas este efeito não foi visto no grupo abstido;
- ✓ Não houve efeito significativo na regulação transcricional de AChE, sugerindo que a diminuição da atividade enzimática está relacionada a mudanças pós-translacionais ou pós-transcricionais;
- ✓ Não houve alterações nas respostas nociceptivas basais pelo teste de tail flick, quando avaliada a funcionalidade dos receptores de adenosina do tipo A_1, observou-se que o grupo cafeína teve sua resposta diminuída ao agonista adenosinérgico (CPA) e o grupo abstido teve este efeito revertido parcialmente demonstrando que este efeito não e de longa duração;

Nossos resultados demonstram que o uso de cafeína durante a gestação e lactação pode trazer prejuízos ao desenvolvimento da prole, salientando a importância da restrição de alimentos e preparações que contenham esta substancia durante os períodos gestacional e de lactação.

8 PERSPECTIVAS FUTURAS

Com base nos resultados e conclusões obtidos com esta dissertação, tem-se como perspectiva avaliar:

- ✓ Consumo hídrico/alimentar de ratas tratadas com cafeína durante a gestação e lactação e ganho de peso das mesmas; Possíveis efeitos teratogênicos na prole ganho de peso dos mesmos.
- ✓ Avaliação de respostas nociceptivas na prole por meio de testes de placa quente e alodinia mecânica utilizando aparato de filamentos de Von Frey.
- ✓ Expressão de receptores de ACh em hipocampo de prole de ratas tratadas com cafeína cronicamente (Artigo 1).
- ✓ Avalição do comportamento materno de ratas tratadas com cafeína durante a gestação e lactação;
- ✓ A funcionalidade de receptores adenosinérgicos por meio da administração de agonistas e antagonistas A_{2A};
- ✓ Os níveis estruturais de BDNF e TNF em hipocampo da prole;

9 DIVULGAÇÕES

a) SOUZA, A. C. ; SOUZA, A. ; Oliveira, C ; ADACHI, L. N. S. ; MEDEIROS, L. F. ; SCARABELOT, V. L. ; SILVA, F. R. ; CAUMO, W. ; SILVA, R. S.; Torres, I.L.S. **Impacto da exposição materna à cafeína sobre a evolução da atividade motora da prole em ratos wistar.** Na XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, 2012, Águas de Lindóia - SP. Anais da XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, 2012.

b) SILVA, F.R. ; SOUZA, A. C. ; SOUZA, A. ; OLIVEIRA, C. ; ADACHI, L. S. ; MEDEIROS, L. F. ; SCARABELOT, V. L. ; CAUMO, W. ; SILVA, R. S. ; TORRES, I. L. S. **Estudo preliminar: exposição materna a diferentes dosagens de cafeína pode resultar em deformidades na prole.** Na 32ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2012, Porto Alegre-RS. Anais da 32ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2012.

c) SOUZA, A. C. ; SOUZA, A. ; OLIVEIRA, C. ; ADACHI, L. S. ; MEDEIROS, L. F.; SCARABELOT, V. L. ; SILVA, F.R. ; CAUMO, W. ; TORRES, I. L. S. **Exposição crônica à cafeína em período gestacional de ratas altera a evolução da atividade motora da prole**. Na 32ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2012, Porto Alegre-RS. Anais da 32ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2012.

d) SILVA, F.R. ; SOUZA, A. C. ; SOUZA, A. ; OLIVEIRA, C. ; ADACHI, L. S. ; MEDEIROS, L. F. ; SCARABELOT, V. L. ; TORRES, I. L. S. **O impacto da**

exposição materna a diferentes dosagens de cafeína e o aparecimento de deformidades na prole em ratos Wistar: estudo preliminar. No XLVII Congresso Anual da Sociedade Brasileira de Fisiologia (SBFis), 2012, Gramado-RS. Anais do XLVII Congresso Anual da Sociedade Brasileira de Fisiologia (SBFis), 2012.

e) SCARABELOT, V. L. ; SOUZA, A. C. ; SOUZA, A. ; OLIVEIRA, C. ; SILVA, F.R.; ADACHI, L. S. ; SILVA, R.S.; TORRES, I. L. S. **Avaliação da evolução da atividade motora da prole de ratas expostas cronicamente à cafeína na gestação.** No XLVII Congresso Anual da Sociedade Brasileira de Fisiologia (SBFis), 2012, Gramado-RS. Anais do XLVII Congresso Anual da Sociedade Brasileira de Fisiologia (SBFis), 2012.

10 ANEXOS

10.1 Aprovação do Comitê de Ética

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

A Comissão Científica e a Comissão de Ética no Uso de Animais (CEUA/HCPA) analisaram o projeto:

Projeto: 110034 **Versão do Projeto:** 04/03/2011

Pesquisadores:
ANDRESSA DE SOUZA
JOANNA RIFOLI ROZISKY
ANA CLÁUDIA DE SOUZA
CARLA DENISE BONAN
ROSANE SILVA DE SOUZA
IRACI LUCENA DA SILVA TORRES

Título: IMPACTO DO TRATAMENTO GESTACIONAL E NEONATAL COM CAFEÍNA NA NOCICEPÇÃO, ATIVIDADE E EXPRESSÃO DE ENZIMAS E DE RECEPTORES DO SISTEMA PURINÉRGICO.

Este projeto foi APROVADO em seus aspectos éticos e metodológicos de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08/10/2008, que estabelece procedimentos para o uso científico de animais.

- Os membros da CEUA/HCPA que participaram do processo de avaliação do projeto onde constam como pesquisadores.
- Toda e qualquer alteração do Projeto deverá ser comunicada à CEUA/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEP/HCPA.

Porto Alegre, 14 de abril de 2011

Dr. Ananda Bender Osvaldt
Coordenador da CEUA/HCPA

10.2 Artigos científicos publicados em co-autoria durante o período de mestrado

a) SOUZA, A. ; MEDEIROS, A.R. ; SOUZA, A. C. ; WINK, M. R. ; SIQUEIRA, I. R. ; HIDALGO, M.P. ; FERNANDES, L. C. ; FERREIRA, M. B. C.; TORRES, I. L. S. Avaliação do impacto da exposição a agrotóxicos sobre a saúde de população rural. Vale do Taquari (RS, Brasil). Ciência & Saúde Coletiva (Online), v. 16, p. 3519-3528, 2011.

b) MEDEIROS, L. F. ; SOUZA, A. C. ; SOUZA, A. ; CIOATO, S.G.; SCARABELOT, V. L. ; CAUMO, W. ; FERNANDES, L. C. ; TORRES, I. L. S. . Fentanyl administration in infant rats produces long-term behavioral responses. Int J Dev Neurosci. 2012; 1:25-30.