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Laura Hautrive Milanesi

INFLUÊNCIA DO CONSUMO DE GORDURA INTERESTERIFICADA NOS PERÍODOS INICIAIS DO DESENVOLVIMENTO SOBRE PARÂMETROS DE ADIÇÃO POR MORFINA EM RATAS

Santa Maria, RS
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Dissertação apresentada ao Programa de Pós-graduação em Farmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Farmacologia**.

Orientadora: Profª. Drª. Marilise Escobar Burger

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Aprovado em 17 de fevereiro de 2017:

Marilise Escobar Burger

Marilise Escobar Burger (UFSM)
(Orientador)

Cristiani Folharini Bortolatto

Cristiani Folharini Bortolatto (UFPEL)

Gabriela Trevisan.

Gabriela Trevisan dos Santos (UFSM)

Santa Maria, RS
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RESUMO

INFLUÊNCIA DO CONSUMO DE GORDURA INTERESTERIFICADA NOS PERÍODOS INICIAIS DO DESENVOLVIMENTO SOBRE PARÂMETROS DE ADIÇÃO POR MORFINA EM RATAS

AUTORA: Laura Hautrive Milanesi
ORIENTADORA: Profª. Drª. Marilise Escobar Burger

Atualmente existe um alto consumo de alimentos industrializados contendo gordura interesterificada (GI), a qual tem substituído a gordura *trans*, desde que esta última mostrou efeitos nocivos à saúde. A morfina é um fármaco opioide amplamente utilizado no alivio da dor aguda e crônica, apresentando alto potencial de dependência e um crescente uso inadequado. Considerando que os fosfolipídeos das membranas neurais podem ser modificados pelos ácidos graxos provenientes da dieta, até os dias de hoje, nenhum estudo avaliou possíveis influências do consumo de GI sobre funções neuronais, envolvendo especialmente o sistema opioide e a morfina, que se tornou o objetivo deste estudo. Ratas *Wistar* foram suplementadas com óleo de soja (OS) ou GI durante todo o período gestacional e de lactação, cujas ninhadas foram mantidas com a mesma suplementação materna até o dia pós natal (DPN) 38. No DPN 39, os filhotes fêmeas foram submetidos ao protocolo de preferência condicionada de lugar (PCL) com morfina, teste do labirinto em cruz elevado e da placa quente. Após as avaliações comportamentais, a imunoreatividade de marcadores moleculares foi avaliada no hipocampo e medula espinhal dos animais. O grupo OS apresentou preferência pela morfina no teste de PCL e sintomas de ansiedade com a retirada da droga, enquanto o grupo GI não mostrou qualquer preferência ou ansiedade. Além disso, o grupo GI apresentou maior sensibilidade ao estimulo térmico. Adicionalmente, os animais do grupo OS que receberam morfina apresentaram um aumento da imunorreatividade do receptor dopaminérgico D1 e glutamatérgico N-metil-D-Aspartato no hipocampo, enquanto estas alterações não foram observadas no grupo GI. Independentemente do condicionamento com morfina, o grupo GI também mostrou imunorreatividade aumentada dos receptores kappa opioides da medula espinhal, cujos dados mostraram correlação negativa com a sensibilidade térmica (r^2=0.67). Os achados aqui apresentados indicam que o consumo crônico de GI durante os períodos iniciais de desenvolvimento pode afetar o sistema de neurotransmissão opioide, modificando as respostas de gratificação relacionadas a este sistema. Considerando que o sistema opioide endógeno está fortemente envolvido nas múltiplas respostas hedônicas fisiológicas, como também na resposta farmacológica de drogas opioides, a continuidade deste estudo é necessária.

Palavras-chave: Ácidos graxos. Sistema dopaminérgico. Adição. Opioides. Hiperalgesia. Fêmeas.

ABSTRACT

INFLUENCE OF INTERESTED FAT CONSUMPTION IN THE INITIAL DEVELOPMENT PERIODS ON MORPHINE ADDICTION PARAMETERS IN RATS

AUTHOR: Laura Hautrive Milanesi
ADVISOR: Profª. Drª. Marilise Escobar Burger

There is a high consumption of processed foods with interesterified fat (IF), which has replaced *trans* fat, given that it has shown harmful effects on health. Morphine is an opioid drug, widely used to alleviate acute and chronic pain, presenting high adicctive potential and increasing inadequate use. Considering that the phospholipids of the neural membranes can be modified by dietary fatty acids, to date, no study has evaluated possible influence of IF consumption on neuronal function, especially involving the opioid system and morphine, which became the aim of this study. *Wistar* rats were supplemented with soybean oil (SO) or IF during the gestational period and lactation, whose pups were maintained with the same maternal supplementation until the postnatal day (PND) 38. On PND 39, the animals were submitted to morphine-induced conditioned preference (CPP) protocol, elevated plus maze and hot plate tests. After the behavioral assessments, the immunoreactivity of molecular markers was evaluated in the hippocampus and spinal cord of the animals. The SO group showed morphine preference in the CPP test and anxiety symptoms with morphine withdrawal while the IF group showed no preference or anxiety. In addition, the IF group presented greater sensibility to thermal stimulation. Besides, animals in the SO group receiving morphine showed increased dopamine D1 receptor and N-methyl-D-Aspartate receptor immunoreactivity in the hippocampus, whereas these changes were not observed in the IF group. Regardless of the morphine conditioning, the IF group showed increased kappa opioid receptors immunoreactivity in the spinal cord, which also showed a negative correlation with the thermal sensibility (r^2=0.67). The findings here indicate that the chronic consumption of IF during the early stages of development may affect the opioid neurotransmission system, modifying the rewards responses related to this system. Considering that the endogenous opioid system is strongly involved in multiple physiological hedonic responses, as well as in the pharmacological response of opioid drugs, the continuity of these studies would be necessary.

Key words: Fatty acids. Dopaminergic system. Addiction. Opioids. Hyperalgesia. Females.

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LISTA DE ABREVIATURAS

AA	Ácido araquidônico
AGE	Ácido graxo essencial
AGPI	Ácido graxo poliinsaturado
AGS	Ácido graxo saturado
AGT	Ácido graxo *trans*
AMPc	Adenosil monofosfato cíclico
D1R	Receptor dopaminérgico 1
DHA	Ácido decosahexaenóico
EPA	Ácido eicosapentaenóico
GI	Gordura interesterificada
KOR	Receptor kappa opioid
LCE	Labirinto em cruz elevado
NMDAR	Receptor N-Metil D-Aspartato
OS	Óleo de soja
PCL	Preferência condicionada de lugar
PKA	Proteína quinase A
SNC	Sistema nervoso central
TG	Triacilglicerol

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APRESENTAÇÃO

Esta dissertação está estruturada em seções dispostas da seguinte forma: **Introdução, Desenvolvimento, Objetivos, Manuscrito científico, Conclusões, Perspectivas e Referências.**

Os itens **Materiais e Métodos**, **Resultados**, **Discussão** e **Referências** encontram-se inseridos no manuscrito, contido na seção **MANUSCRITO CIENTÍFICO,** representando a íntegra deste estudo.

As **REFERÊNCIAS** dizem respeito somente as citações que aparecem no item **INTRODUÇÃO** e **DESENVOLVIMENTO** desta dissertação.

1. INTRODUÇÃO

As alterações na composição dos ácidos graxos da dieta estão envolvidas na regulação de várias atividades celulares, podendo levar a diversas condições patológicas como obesidade (AILHAUD et al., 2004), resistência à insulina (BORKMAN et al., 1993), hipertensão e doença cardiovascular (CLARKE, 2009; MOZAFFARIAN; ZHENG et al., 1999). O consumo de gordura *trans* está associada a um aumento do risco de doenças neuropsiquiátricas (TEIXEIRA et al., 2012) e parâmetros de dependência (KUHN et al., 2013, 2015, ROVERSI et al 2016), que possivelmente estão relacionados a alterações na neurotransmissão dopaminérgica (ACAR et al., 2003).

Em razão dos diversos efeitos deletérios a saúde, a gordura *trans* está sendo reduzida ou até mesmo eliminada da produção de alimentos (UPRITCHARDA et al., 2005), sendo atualmente substituída pela gordura interesterificada (GI) (L'ABBE et al., 2009). A interesterificação é um processo utilizado pela indústria para produção de gorduras com propriedades físico-químicas melhoradas (RONNE et al., 2005). Este processo envolve o re-arranjo dos ácidos graxos entre e dentro das moléculas de triglicerol (TG) (ROBINSON, et al., 2009).

Os opioides são muito empregados no controle da dor moderada a grave, porém seu uso é dificultado pelo desenvolvimento de tolerância após sua administração a médio e longo prazo (OSSIPOV et al., 2004). Também, o uso não-médico de opioides prescritos é crescente tanto em jovens como adultos e é considerado um problema de saúde pública (SHARMA et al., 2016). Estudos do nosso grupo demonstraram um aumento da preferência pela morfina causado pelo consumo de gordura *trans* (ROVERSI et al., 2016) porém ainda não foi demonstrado o efeito do consumo de GI sobre parâmetros de adição a morfina bem como o efeito deste consumo no sistema nervoso central (SNC), o que torna o objetivo do nosso estudo. Descobrir a ligação e os possíveis mecanismos da dieta à tendência ao abuso de drogas é de extrema importância visto que poderá resultar em uma estratégia de prevenção para um problema de saúde pública, bem como alertar sobre problemas relacionados a dieta atual.

2. DESENVOLVIMENTO

2.1 Ácidos graxos atualmente consumidos

Os óleos e gorduras da dieta têm um importante papel nutricional, sendo fonte de calorias, ácidos graxos essenciais (AGE), conferindo também maior palatabilidade aos alimentos (FENNEMA, 2010). Ácidos graxos essenciais (AGE) são importantes constituintes de todas as membranas fosfolipídicas e a substituição destes AGE nas membranas podem alterar a sua fluidez bem como o funcionamento de enzimas, receptores, transportadores e a plasticidade sináptica (JUMP et al., 2002). Os AGE não são sintetizados no corpo humano, sendo obtidos através da dieta (DAS, 2008), e classificados em duas classes principais, ômega-6 e ômega-3 (SIMOPOULOS, 2016).

Atualmente, a dieta consumida pela população contêm altos níveis de ômega-6 e baixos níveis de ômega-3, o que eleva a razão ômega-6/ômega-3 para 20:1, ao invés de 1:1 (KANG, 2003; SIMOPOULOS, 2008), a qual é considerada a razão ideal para a saúde humana. Um desequilíbrio na proporção omega-6/omega-3 a favor do omega-6 é altamente pró-trombótico e pró-inflamatória, o que contribui para a prevalência da aterosclerose, obesidade, e diabetes (DONAHUE et al., 2011; KROMHOUT et al., 2014; SIMOPOULOS, 2013). Há indícios crescentes que apoiam os efeitos benéficos de um aumento da ingestão de ômega-3, exibindo propriedades neuroprotetoras em uma variedade de condições neurológicas e neurodegenerativas (DYALL; MICHAEL-TITO 2008; DYALL et al., 2010; DENIS et al., 2015).

Quimicamente, as gorduras são formadas pela união de três ácidos graxos com uma molécula de glicerol, conhecendo-se essa molécula com o nome de triglicerídeos ou triacilgliceróis (TG). As moléculas de TG podem ser sólidas ou líquidas em temperatura ambiente, dependendo de sua estrutura e de sua composição. Usualmente o termo "gordura" se refere aos TG em seu estado sólido, enquanto que o termo óleo, aos TG no estado líquido (KODALI; LIST, 2006).

A gordura vegetal parcialmente hidrogenada foi pioneiramente empregada na produção de alimentos industrializados, já que a mesma é rica em ácidos graxos *trans* (AGT), os quais são responsáveis pela maior consistência, palatabilidade e maior prazo de validade dos mesmos. No entanto, devido a efeitos prejudiciais para a saúde (MOZAFARIAN; CLARKE, 2009; SUN

et al., 2007), a legislação de vários países, incluindo o Brasil, colocou restrições sobre o emprego de AGT na produção de alimentos (RATNAYAKE et al., 2014).

O consumo regular de AGT pode, eventualmente, resultar em uma perda de AGE, podendo alterar a plasticidade e neurotransmissão (LARQUÉ et al., 2003). A ingestão de AGT também tem sido associada à disfunção cognitiva (MORGAN et al., 2007; YAFFE et al., 2007), alterações na neurotransmissão dopaminérgica (ACAR et al., 2003), a dependência (KUHN et al., 2013), mania (TREVIZOL et al, 2011), perturbações do movimento (TEIXEIRA et al., 2012) e uma maior sensibilidade a drogas de abuso (ROVERSI et al., 2016). Atualmente, a gordura *trans* está sendo substituída pela gordura interesterificada (GI) na produção de alimentos (AFONSO et al., 2016).

2.2 Gordura Interesterificada

Os processos tecnológicos de modificações de óleos e gorduras é de grande interesse industrial uma vez que tem por objetivo obter gorduras com propriedades como fusão e cristalização adequadas e estes processos incluem por exemplo: a interesterificação (SENANAYAKE; SHAHIDI, 2005). A gordura interesterificada (GI) resulta da modificação da posição dos ácidos graxos na molécula de glicerol, formando um novo TG (BERRY et al., 2009).

A técnica de interesterificação consiste em uma gordura e um óleo, ou duas gorduras submetidas a um processo de rearranjo na molécula de TG. Esse processo é onde dois ou mais óleos e/ou gorduras são misturados para se obter um produto com composição e consistência desejáveis (GUNSTONE, 1998; SENANAYAKE; SHAHIDI, 2005). Os produtos obtidos desta forma poderão conter mais ácidos graxos saturados (AGS) porém sem a presença de AGTs (GUNSTONE, 1998).

Os TG possuem um comportamento de fusão adequado o que contribui para a formação de um produto com textura desejável. A fusão de óleos e gorduras é resultado de uma interação entre as moléculas de TG e dos diferentes AG presentes nela. Nesse sentido, a modificação da posição dos AG na molécula de TG pode resultar em alterações nas propriedades de fusão deste (RIBEIRO et al., 2007). No processo de interesterificação não há mudança na composição de AG, somente na composição dos TG, o que leva à modificação das propriedades físicas (AHMADI; MARANGONI, 2009).

A interesterificação (randomização de ácidos graxos) da gordura está se tornando rapidamente uma alternativa, em que a inserção de AGS, tipicamente na forma de ácido esteárico, é empregada para endurecer um óleo para uma plasticidade comparável a gordura *trans* ou a dureza de uma gordura saturada natural (WARNICK et al., 1982). A interesterificação provém da troca de moléculas de ácidos graxos entre si e entre as moléculas de TG, resultando em espécies moleculares de TG alteradas. Estas alterações causam modificações nas suas propriedades físico-químicas (REENA et al., 2009). Tais alterações foram relatadas por influenciar na digestão e absorção destas gorduras (SMALL et al., 1991) e causar significativa alterações nas suas propriedades nutricionais (RAO et al., 2003).

Existem dois processos para a obtenção de GI: enzimático e químico. No processo enzimático é utilizado lipases microbianas enquanto na interesterificação química utiliza-se catalizadores como, por exemplo, metóxido de sódio (MeONa) (RIBEIRO et al., 2007). A interesterificação química é a mais utilizada pela indústria e possui algumas vantagens em relação ao método enzimático, uma vez que ela é uma alternativa mais barata (MARANGONI; ROUSSEAU, 1995) e mais rápida (ALLEN et al., 1996) quando comparado as lipases.

Alguns autores mostraram que o consumo de GI pode aumentar os riscos metabólicos envolvidos no diabetes tipo 2, doença cardiovascular (ROBINSON et al., 2009) e aterosclerose (AFONSO et al., 2016). Um estudo realizado por Bispo e colaboradores (2015) demonstrou que o consumo de GI no período de desenvolvimento (gestação e lactação) resultou no comprometimento do efeito da insulina no centro hipotalâmico, responsável por inibir a ingestão de comida (BISPO et al., 2015).

2.3 Gorduras em períodos de desenvolvimento

Diversos estudos indicam que o desenvolvimento do cérebro pode ser influenciado pela dieta, especialmente nas fases iniciais da vida, quando os eventos de plasticidade sináptica e reorganização neuronal são mais intensos (FERNANDES et al., 2011; de VELASCO et al., 2012; MURPHY et al., 2014). Sendo assim, a dieta materna durante os períodos críticos (gestação e lactação) podem impactar no desenvolvimento cerebral, funcionando como um fator epigenético que pode influenciar em alterações metabólicas na vida adulta (MISAN et al., 2015). Nos últimos anos, as pesquisas que investigam os efeitos do excesso de ingestão de lipídios na dieta materna, forneceu algumas evidencias de que a manipulação dietética lipídica

perinatal resulta em pronunciadas disfunções metabólicas no decorrer da vida adulta (KRASNOW et al., 2011; SULLIVAN et al., 2011).

Os ácidos graxos passam de mãe para feto através da barreira placentária, e após o nascimento, através do leite materno. Sabe-se que é durante o último trimestre de gestação e os primeiros 18 meses de vida pós-natal humana e 15 dias pós-natal em ratos (DOBBING; SANDS, 1979) que ocorre a maior taxa de incorporação de ácidos graxos do sistema nervoso central e é nesse período que ocorre a maior fase do crescimento cerebral (CARLSON, 2001; DIJCK-BROUWER et al., 2005).

Estudos recentes tem demonstrado que a ingestão de AGT durante o período de lactação resulta em importantes modificações metabólicas culminando na redução da produção de lipídeos nas glândulas mamárias, dos quais são de extrema importância para a lactação (ASSUMPÇÃO et al., 2002). Roversi e colaboradores (2016) demonstraram que a suplementação de ratos com AGT durante os períodos iniciais da vida aumentou a preferência por opióide (ROVERSI et al., 2016).

2.4 Adição a opioides

O sistema opioide é crítico na modulação do comportamento de dor e de antinocicepção (MANCHIKANTI et al. 2012). Peptídeos opioides e os seus receptores são expressos em todo o circuito neural além de regiões críticas incluíndo regiões de recompensa e estruturas cerebrais relacionadas com a emoção (MORALES et al., 2016). Existem três diferentes tipos de receptores opioides (mu (μ), delta (δ), Kappa (κ)) sendo eles acoplados a uma proteina G inibitória a qual inibe a formação de adenosil-monofosfato cíclico (AMPc). Uma vez que a ativação de receptores opioides inibe a atividade de adenilato-ciclase, ocorre uma redução do AMPc, bem como a atividade da proteína quinase A (PKA) (DHAWAN et al., 1996).

Ao longo das últimas duas décadas, o uso de drogas opioides aumentou em dores crônicas não-cancerosas (MANCHIKANTI et al., 2012). Também tem sido mostrado que apenas um em seis, ou 17,3% dos usuários de opioides não-terapêuticos, relataram que receberam os fármacos através de prescrição médica (NSDUH) (US Department of Health and Human Services, 2015). Morfina, oxicodona e meperidina, além de heroína, o que é ilegal na maioria dos países, são os opioides mais comumente utilizados com abuso, e seu uso contínuo favorece o desenvolvimento de dependência e tolerância (POULETTY et al., 2002; YARGEAU et al., 2014).

A morfina é clinicamente utilizada no alívio de dor moderada a grave, mas sua eficácia é gradualmente perdida com o rápido desenvolvimento de tolerância (MAYER et al. 1999). A tolerância é caracterizada por um estado diminuído da responsividade ao efeito da droga, sendo necessário o aumento da dose para a obtenção dos mesmos efeitos iniciais (CHU et al., 2012). A tolerância é um quadro comum observado em usuários de opioides, e os mecanismos moleculares subjacentes a estes efeitos incluem alterações adaptativas em diferentes circuitos neurais, incluindo a dessensibilização e internalização dos receptores opioides (UEDA, et al., 2009). Com administração crônica de opioides ocorre além do desenvolvimento de tolerância analgésica, ocorre uma hiperalgesia induzida pela retirada da droga, limitando sua eficácia clínica (JIN et al., 2016).

É bem aceito que a plasticidade neural tanto no cérebro como na medula espinhal desempenha um papel importante na tolerância analgésica da morfina. O uso crônico da morfina leva a ativação das células gliais (microglia e astrócitos) contribuindo no desenvolvimento desta tolerância (CHEN; SOMMER, 2009) uma vez que esta ativação leva a um aumento da produção de substâncias como citocinas pró-inflamatórias e aminoácidos excitatórios (HAN et al., 2014). Outra mudança na plasticidade, decorrente do uso crônico de morfina, envolve a ativação de receptores de aminoácidos excitatórios como o receptor N-metil-D-aspartato (NMDA) (LIM et al., 2005). Os receptores NMDA possuem um papel importante no desenvolvimento de hiperalgesia e tolerância induzidos pela morfina. Já foi demonstrado que o antagonista dos receptores NMDA (MK-801) impediu o desenvolvimento de tolerância induzido pela morfina (MAO, et al., 1992).

Além disso, o uso crônico de morfina leva à dependência física e psicológica, e sua retirada desencadeia sintomas aversivos conhecidos como síndrome da abstinência (O'BRIEN et al., 1997). Os opioides têm um alto poder aditivo, levando o indivíduo adicto a perda do controle emocional e ao comportamento compulsivo, resultando em conseqüências sociais e sobre a saúde (HYMAN et al., 2006; MILTON; EVERITT, 2012). A retirada de opioides é caracterizada por ansiedade, hipersensibilidade à dor e busca por drogas (ZHANG et al., 2016), que estão associadas ao estado emocional negativo e contribuem para a recaída após períodos de abstinência (KOOB; Le MOAL, 2005). Além disso, a retirada da morfina é caracterizada pelo aumento dos níveis de cortisol no sangue que são indicativos de estresse e ansiedade (ROY et al., 2006) Estudos indicam que tanto a ansiedade aumentada como o processamento hedônico alterado, ou seja, uma redução da motivação natural, são vistos quando ocorre a retirada da droga (ASTON-JONES; HARRIS, 2004).

O sistema dopaminérgico é um alvo bem conhecido de drogas com potencial aditivo e a plasticidade dentro deste circuito neural é que está envolvida no desenvolvimento desta adição (HYMAN et al., 2006; KAUER; MALENKA, 2007). A atividade dos neurônios dopaminérgicos é fortemente regulada pela neurotransmissão excitatória glutamatérgica e inibitória gabaérgica, desde que alterações na plasticidade destes sistemas ocorre desde a primeira exposição a drogas de abuso (BAIMEL; ORGLAND, 2015).

A administração aguda de morfina produz excitação significativa em neurônios dopaminérgicos, enquanto que a exposição prolongada ou repetida da droga provoca alterações adaptativas na função celular e sináptica deste mesmo sistema (NESTLER et al., 2001). Um paradigma amplamente aceito para a adição decorrente do uso da morfina, indica que ela excita neurônios dopaminérgicos por redução da transmissão gabaérgica através da ativação de receptores opioides do tipo μ (FORD et al, 2006; JOHNSON; NORTH 1992; SHOJI et al., 1999). Os efeitos da morfina em neurônios dopaminérgicos foram completamente revertidos pela administração sistêmica do antagonista μ-opioide, naloxona (LIU et al., 2015). Sendo assim, os efeitos de recompensa, bem como os de memória relacionada com os opioides é produzido através da neurotransmissão dopaminérgica (LAVIOLETTE et al., 2002).

Já está descrito na literatura que a diferença entre gêneros influencia parâmetros como a preferência por drogas, incluindo dos opioides. As mulheres se tornam adictas as drogas de abuso mais rapidamente, assim como apresentam os sintomas de síndrome da abstinência mais graves quando comparadas aos homens (BACK et al., 2011; BRADY; RANDALL, 1999; IGNJATOVA; RALEVA, 2009). Estudos experimentais demonstraram que as fêmeas são mais suscetíveis ao efeitos aditivos das drogas de abuso como os opioides bem como ao desequilíbrio na dieta rica em gordura (KARAMI; ZARRINDAST, 2008; STERLING et al., 2004).

A sensibilidade dos receptores opioides a lipídios já foi documentada. O tratamento crônico de células neurais com ácidos graxos livres reduziu a capacidade de ligação dos agonistas opioides em seus receptores (HO; COX, 1982; McGEE; KENIMER, 1982). Como mostrado por Remmers e colaboradores (1990), dependendo do perfil do ácido graxo, diferentes respostas nas funções dos receptores opioides pode ser esperada. Mudanças no perfil dos ácidos graxos induzido pela dieta, idade e distúrbios fisiopatológico podem alterar a interação dos receptores opioides com o sistema opioide endógeno (REMMERS et al., 1990).

2.5 Paradigma de Preferência condicionada de lugar

Como descrito anteriormente, a morfina é um analgésico opioide amplamente utilizado para o alívio da dor mas seu uso prolongado está relacionado a dependência física e psicológica (HYMAN et al. 2006; MILTON; EVERITT 2012). Estes efeitos de reforço dos opiáceos responsáveis pela adição são conhecidos tanto em humanos como em animais experimentais (LESHNER; KOOB 1999). O paradigma de preferência condicionada de lugar (PCL) é usado em animais experimentais para avaliar os efeitos de recompensa de drogas com potencial aditivo, incluindo a morfina (TZSCHENTKE, 2007).

Este paradigma baseia-se no pareamento ou associações dos estímulos contextuais e as propriedades de recompensa da droga (RIBEIRO et al., 2005) e tem sido usado para o estudo dos mecanismos neuroquímicos envolvidos na recompensa e desenvolvimento da adição (LIN et al., 2014). O desenvolvimento dessas associações está relacionado a mudanças na plasticidade em sistemas que regulam a recompensa e também são responsáveis pela formação de uma memória (DONG; NESTLER, 2014).

Para que ocorra a recompensa pela droga e conseqüentemente o desenvolvimento de adição é necessário que haja a aprendizagem e a formação de uma memória relacionada ao estímulo e ao contexto (TORREGROSSA et al., 2011). O paradigma de PCL é baseado em uma associação positiva de um contexto com a droga utilizada, sendo esta associação chamada de condicionamento Pavloviano (SHETTY et al., 2017). O condicionamento clássico Pavloviano associa um "estímulo incondicionado" adquirido em seções repetidas com um "estímulos condicionados" (TZSCHENTKE, 2007). Drogas de abuso levam a uma aprendizagem bem como aumentam o condicionamento Pavloviano (TORREGROSSA et al., 2011). A neurotransmissão dopaminérgica exerce um papel fundamental na formação de memória, sendo que os receptores dopaminérgicos D1 estão envolvidos no condicionamento Pavloviano (SCHULTZ, 2013).

O condicionamento com drogas de abuso induz respostas adaptativas principalmente no hipocampo, estrutura cerebral de grande importância para o armazenamento da memória no caso associativa (MAREN, 2001). O maior obstáculo no tratamento da adição é a recaída e a busca pela droga quando o contexto, o qual foi previamente associado ao uso, é novamente apresentado (CHILDRESS et al., 1999).

2.6 Ansiedade e o labirinto em cruz elevado

A retirada de fármacos opioides, como a morfina, em usuários crônicos, desencadeia alguns sintomas incluindo sintomas de ansiedade (MILADI-GORJI et al., 2011). Estudos em animais demonstraram que tanto a retirada da morfina como o uso de antagonista resulta em aumento do comportamento de ansiedade avaliado no teste de labirinto em cruz elevado (LCE).

Um dos testes comportamentais mais populares para a avaliar a ansiedade em modelos animais é o LCE (LISTER, 1987). O LCE tem sido amplamente utilizado como ferramenta na investigação das bases psicológicas e neuroquímicas da ansiedade, bem como para a triagem de medicamentos que a modulem (BELZUNG; GRIEBEL, 2001). O aparato do LCE é na forma de um sinal positivo, distante do solo, com dois braços abertos e elevados voltados um para o outro e separados por um quadrante central e dois braços das mesmas dimensões, mas fechado por paredes laterais e opostos entre si (MONTGOMERY, 1955).

O teste do LCE é baseado na aversão natural de roedores para entrar e permanecer nos braços abertos, e usa o conflito entre a exploração e a aversão. Os ratos naturalmente mostram um padrão de comportamento que demonstra preferência pelos braços fechados (BOURIN, 2015). Este comportamento, relacionado à esquiva aos braços abertos está relacionado a maior ansiedade, podendo ser induzido por drogas ansiogênicas (CRUZ et al., 1994). O teste do LCE, é uma ferramenta importante para a descoberta de ansiolíticos, uma vez que este efeito é observado com um aumento do tempo de permanência nos braços abertos (PAPP et al., 2006).

2.7 Nocicepção e o teste da placa quente

Estudos mostram uma redução significativa nos efeitos analgésicos da morfina e de outros opioides após a administração repetida. A administração repetida também está associada a um aumento da sensibilidade a estímulos nociceptivos, fenômeno conhecido como hiperalgesia (LEE et al., 2011; LIANG et al., 2006). Haleem e colaboradores (2017) demonstraram que a administração repetida de morfina resultou na hiperalgesia avaliada no teste da placa quente em ratos (HALEEM; NAWAZ, 207)

O teste da placa quente é um modelo primeiramente descrito por Woolfe e Macdonals (1944) com o objetivo de descobrir substâncias com potencial analgésico e de efeito central (WOOLFE; MACDONALD, 1944). O teste da placa quente é utilizado para avaliar o efeito antinociceptivo central mediado via receptores espinhais (NEMIROVSKY et al., 2001). No

aparato, uma chapa metálica aquecida é mantida em temperatura controlada e provoca respostas comportamentais, como pular, remover ou lamber a pata do animal que está sobre ela (ORLANDI et al., 2011). O animal é avaliado quanto a latência de resposta nociceptiva, sendo que a menor latência corresponde a maior resposta de hiperalgesia (WOOLFE; MACDONALD, 1944).

3. OBJETIVOS:

Objetivo geral:

Avaliar a influência do consumo de gordura interesterificada ao longo do desenvolvimento intrauterino e pós-natal (gestação, lactação e pós-desmame) de ratos *Wistar* sobre parâmetros comportamentais e moleculares relacionados ao condicionamento com morfina.

Objetivos específicos:

- Avaliar a influência da suplementação de gordura interesterificada (GI) durante períodos iniciais da vida e durante o desenvolvimento, sobre parâmetros de preferência por morfina;
- Avaliar a influência da suplementação de GI sobre parâmetros comportamentais relacionados à ansiedade conseqüente à retirada da morfina nos mesmos animais previamente condicionados com a droga;
- Avaliar possíveis alterações comportamentais de hiperalgesia ou tolerância analgésica destes animais que foram inicialmente expostos à GI e posteriormente condicionados com morfina;
- Avaliar aspectos moleculares relacionados aos receptores envolvidos nos parâmetros de adição a morfina em áreas cerebrais relacionadas ao comportamento.

4. MANUSCRITO CIENTÍFICO

Os resultados inseridos nesta dissertação apresentam-se sob a forma de manuscrito científico, o qual encontra-se aqui estruturado sob o título: “Chronic consumption of interesterified fat modifies brain opioid system and affects morphine-induced reward effects in rats”. Os itens Materiais e Métodos, Resultados, Discussão e Referências, encontram-se inseridos no próprio manuscrito.

CHRONIC CONSUMPTION OF INTERESTERIFIED FAT MODIFIES BRAIN OPIOID SYSTEM AND AFFECTS MORPHINE-INDUCED REWARD EFFECTS IN RATS

Laura H. Milanesi[a]; Karine Roversi[a]; Caren T. D. Antoniazzi[a]; Hecson J. Segat[b]; Maikel Kronbauer[a]; Lívia F. D'Ávila[a]; Verônica T.Dias[a]; Marcel H. M. Sari[b], Raquel C. S. Barcelos[a]; Luana H. Maurer[c];Tatiana Emanuelli[a,c]; Marilise E. Burger[a,b,c]; Fabíola Trevizol[a]*

[a]Programa de Pos-Graduação em Farmacologia, Universidade Federal de Santa Maria (UFSM), RS,Brazil;
[b]Programa de Pos-Graduação em Bioquímica Toxicologica, UFSM, RS, Brazil
[c]Programa de Pos-Graduação em Ciência e Tecnologia dos Alimentos, UFSM, RS, Brazil

Address of the authors:
laura-milanesi@hotmail.com; karineroversi_@hotmail.com; carenantoniazzi@yahoo.com.br; hecson_segat@hotmail.com; maikel_kr@hotmail.com; liviadavila@hotmail.com; vel_td@hotmail.com; marcelsarih@hotmail.com; raquel.barcelos@hotmail.com; luanahmaurer@gmail.com; tatiana.emanuelli@ufsm.br; marilise.burger@ufsm.br; fatrevizol@yahoo.com.br*

***Corresponding author**:
fatrevizol@yahoo.com.br
Graduation Program of Pharmacology
Universidade Federal de Santa Maria (UFSM)
Santa Maria-RS, 97105-900 Brazil.

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Abstract

Opioid analgesics are intensely used to alliviate acute and chronic pain, presenting high addictive potential and its improper use may be related to addiction, tolerance and withdrawal syndrome. Considering the high consumption of processed foods, interesterified fat (IF) has been used in replacement to *trans* fat, since it causes deleterious health effects and may harm nervous system functions. So far, no studies reported the influence of IF consumption on neuronal function, specially involving the opioid system and morphine, which became the aim of this study. Wistar rats were supplemented with soybean oil (SO) or IF during gestation, lactation and postweaning periods until pups' adolescence. On posnatal day 39, animals were injected with morphine (4mg/kg i.p.) in a conditioned place preference (CPP) paradigm. After morphine-CPP test, anxiety-like symptoms and thermal sensibility were evaluated. Hippocampus and spinal cord were used for molecular markers assessments. SO group showed morphine preference during drug withdrawal while IF group showed no preference or withdrawal symptoms, presenting higher sensibility than SO group to thermal stimuli. In addition, morphine contidioning increased dopamine D1 receptor and N-metyl-D-Aspartate receptor immunoreactivity in the hippocampus of SO group, while these molecular changes were not observed in IF group. Independently of morphine conditioning, IF group showed increased kappa opioid receptors immunoreactivity in the spinal cord, which also showed negative correlation with thermal sensibility. Our findings indicate that chronic consumption of IF-rich foods during earlier periods of life may affect opioid neurotransmission system, resulting in loss of rewarding effects related to this system.

Key words: Fatty acids, Developmental periods, Conditioned place preference, Kappa opioid receptor, D1 receptor, NMDA receptor, Female.

Introduction

Opioid drugs such as morphine are the most effective analgesics used in the clinic to relief different types of pain, but over the years, the opioid use without therapeutic purposes has been increased (Dhalla et al. 2009; Manchikanti et al. 2012). In the last 10 years, the number of opioid prescriptions increased by 48% in U.S. (Manchikanti et al. 2010; Fischer et al. 2014), while its non-medical use affects 4.5 million individuals in 2013 (SAMHSA, 2014). The prolonged use of opioids is recognized by tolerance development (Mao et al. 1995; Mayer et al. 1999), hyperalgesia (Hutchinson et al. 2011; Xin et al. 2012), besides physical and psychological dependence, which are related to withdrawal syndromes (O'Brien et al. 1997). In fact, opioids have a high addictive power, and lead the addict to loss control over intake, resulting in social and health consequences (Hyman et al. 2006; Milton and Everitt 2012). The opioid withdrawal is characterized by anxiety, hypersensitivity to pain and drug seeking (Zhang et al. 2016), which are associate with negative emotional state and contribute to relapse after abstinence periods (Aston-Jones and Harris 2004; Koob and Le Moal 2005). Opioids exert their therapeutic action by binding to μ, k and δ receptors; while μ receptors are involved in pain relief besides tolerance and dependence development, δ receptors are involved in the tolerance development (Bailey and Connors 2005), and, in contrast, *k* receptors have been involved in loss of reward effects produced by morphine, in addition to its analgesic effect (Margolis et al. 2003; Chefer et al. 2005).

It is already described in the literature that lifestyle, especially eating habits, can exerts significant influences on reward pathway that are related to development and maintenance of addiction (Blumenthal et al. 2010; Carter et al. 2016). In this sense, our research group have been unstinting to show that *trans* fatty acids (TFA) from enriched diets can be incorporated into the neural membranes, increasing risk for neuropsychiatric conditions (Teixeira et al. 2011; Trevizol et al. 2011; 2014; Pase et al. 2013; 2015; Dias et al. 2015) thus modifying addiction parameters (Kuhn et al. 2013; 2015; Roversi et al. 2016), which possibly are related to changes in the dopamine neurotransmission (Acar et al. 2003). The consumption of TFA has been associated with harmful effects to health and in view of that, their consumption is not recommended during pregnancy and lactation (Osso et al. 2008; Anderson et al. 2010; Lefevre et al. 2012; Magri et al. 2014). Also, maternal nutrition during pregnancy and lactation determines significant transfer of essential fatty acids through the placenta and milk to the fetus and may interfere with fetal nervous system development (Herrera et al. 2002; Souza et al. 2012). Neuringer et al (1988) pointed out that the greatest absorption of fatty acids (FA) from

the diet by the brain membranes mainly occurs during the initial stages of life. This absorption is related to affect the neural membrane phospholipids composition and thus, influence their structure and neurotransmission (Fernstrom 1999).

The use of TFA has been reduced or eliminated from the processed foods through use of technologies such as interesterification of fats (L'Abbè et al. 2009; Reming et al. 2010; Gagliardi et al. 2013). This interesterification process involves FA randomization through re-arrangement within and between triacylglycerol molecules, by either enzymatic or chemical methods (Robinson et al. 2009) in order to obtain a good yield of desirable melting characteristics (L'Abbè et al. 2009). Interesterification results in improvements in physical and chemical characteristics, which are important for the food industry (Farfán et al. 2013). Some authors showed that the interesterification process may increase metabolic risks involved in type-2 diabetes, cardiovascular disease (Robinson et al. 2009) and atherosclerosis (Afonso et al. 2016). Being a fat currently introduced in processed foods, there are few studies about consequences of their consumption on health during early development, especially on central nervous system functions.

Considering critical periods of life such as gestation and lactation (Pase et al. 2013) and the current dietary habits, that involve a chronic consumption of interesterified fats from processed foods, we aimed to evaluate the influence of such consumption on morphine addictive properties in adolescent pups, which are more susceptible to the rewarding effects of drugs (Badanich et al. 2006; Brenhouse and Andersen 2008; Torres et al. 2008; Zakharova et al. 2009).

Materials and methods

Animals and experimental procedure

Twelve female pregnant *Wistar* rats from the breeding facility of Universidade Federal de Santa Maria (UFSM), RS, Brazil, were individually kept in plexiglas cages with free access to food and water in a room with controlled temperature (22±2ºC) and on 12 h-light/dark cycle with lights on at 7:00 a.m. This study was approved by the Animal Ethical Committee of Universidade Federal de Santa Maria (041235/2016-UFSM), affiliated to the Council for the Control of Animal Experiments (CONCEA), following international norms of animal care and maintenance.

Female pregnant *Wistar* were randomly distributed in two experimental groups (n=12) according to oral supplementation: Soybean oil (SO, rich in n-6 FA; Camera®, Santa Rosa-RS,

Brazil, purchased at local supermarket), which was used as control group, considering its high consumption worldwide (Zhang et al. 2012); and interesterified fat (IF, Triângulo Allimentos LTDA, Itópolis-SP, Brazil). The profile of fatty acids present in each supplementation was determined by gas chromatography (Hartman and Lago 1973) (Table 1). Animals were orally supplemented (3g/kg, p.o. once a day) (Trevizol et al. 2011; Kuhn et al. 2013; Pase et al. 2013; Dias et al. 2015) during gestation and lactation (totaling 43 days). After weaning, female pups of each litter were maintained in the same maternal supplementation until adolescence period, on the postnatal day (PND) 38.On PND 39, animals were assigned in four experimental groups, considering their previous supplementation: SO-vehicle, SO-morphine, IF-vehicle, IF-morphine; (n=6 each group), and were exposed to the behavioral assessments as described below (Fig. 1).

Our experimental protocol was performed with female pups following previous reports, which showed that female rats are more susceptible to imbalance in fat diet and drug reward addictive effects as opioid drugs (Sterling et al. 2004; Karami and Zarrindast 2008; Jen et al. 2009; Roversi et al. 2016).

Drugs and solutions

Morphine sulphate (São Paulo, Brazil) was diluted in 0.9% NaCl solution and injected intraperitoneally (i.p.) in a dose of 4 mg/kg (Vey et al. 2015; Roversi et al. 2016). Vehicle injections were 0.9% NaCl solution.

Conditioned place preference (CPP) procedure

The CPP procedure is a well-established behavioral paradigm that has been widely employed to assess symptoms of craving, reward, extinction and relapse to addictive drugs (Kuhn et al. 2013; Segat et al. 2014; Antoniazzi et al. 2014a; 2014b; Vey et al. 2015).It uses a three compartment box separated by manual guillotine doors: two compartments of equal size (45 cm × 45 cm × 50 cm) with equivalent intensity of light but different visual clues: one with a white floor and striped walls, and other with a striped floor and smooth white walls. These two compartments converge to a third smaller compartment. The apparatus was cleaned with alcohol 20% using wet sponge and paper towel before the introduction of each animal. CPP methods have been described in detail previously (Shi et al.2004; Thanos et al. 2010) and was performed through the following steps: habituation, pre-test, conditioning, and test. On day 1, rats (PND 39) were kept for 15 min in each compartment for habituation. The aim of this

procedure is to eliminate exploratory behavior, which is common in new environments, for both pre-testing and conditioning phases, thereby avoiding misinterpretations. On day 2 we performed the pre-test, which consists in letting the animal freely choose one of the compartments for 15 min. Animals showing some natural preference were excluded from the experiment. On the next 4 days, animals were conditioned with morphine (4 mg/kg i.p.) for 30 min in the non-preferred compartment, and with vehicle in the preferred compartment, with an interval of 4 h between each administration. The vehicle group received 0.9% NaCl injections on both sides of the apparatus. After the4-day conditionings, rats were placed at the center choice chamber with access to the entire apparatus, for 15 min to perform the CPP test (PND 45). Time spent in the drug-paired environment was interpreted as preference, whereas time spent away from that environment was interpreted as aversion.

Elevated plus maze (EPM) task

To evaluate the influence of supplementation on the anxiety-like symptoms, animals were observed in the EPM, which is based on the innate fear rodents have for open and elevated spaces (Montgomery, 1955). The apparatus consists in a platform elevated 50 cm from the floor. Forty-centimeter (40 cm) high walls enclose two opposite arms (50 cm × 10 cm) whereas the other two arms have no walls. All arms have a central intersection (10 cm × 10 cm). At the test beginning, the rat was placed in central intersection facing an open arm. The entries number and time spent in the open arms were quantified for 5 min in the EPM. We also calculated the anxiety index, which integrates the EPM behavioral measures as demonstrated in the following formula:

$$\text{Anxiety index} = 1 - \left[\frac{\left(\frac{\text{Tempo gasto}}{\text{Tempo total}}\right) + \left(\frac{\text{Número de entradas}}{\text{Número total}}\right)}{2}\right]$$

Anxiety index values range from 0 to 1, where an increase in the index expresses increased anxiety-like behavior (Cohen et al. 2007; Mazor et al. 2007). The apparatus was cleaned with alcohol solution (20%) and dried with paper towel before the introduction of each animal.

Assessment of thermal sensibility with the hot plate test

Thermal sensibility was assessed on the hot plate test, according to a method previously described (Woolfe and MacDonald 1944). In this test, animals were placed in the hot plate

apparatus (Insight® EFF- 361), and a hot thermal stimulus on a metal plate (55ºC ±0.5ºC) was used to determine their hyperalgesia. The latency for removal or licking the paws was recorded. If the animals did not respond within 45 sec (cut-off time), they were removed from the plate to avoid tissue damage. Where in a lower latency corresponds to increased hyperalgesia. After the hot plate test, animals were returned to the experimental cages.

Immunoblotting

One day after the last behavioral assessment, animals were anesthetized (sodium pentobarbital, 50mg/kg body weight i.p.) and euthanized by cervical decapitation. Their brains were removed and cut coronally at the caudal border of the olfactory tubercle to remove the hippocampus (Paxinos and Watson2007). Hippocampal and spinal cord tissues were homogenized with a lysis buffer containing 137 mM NaCl, 20 mM Tris–HCl pH 8.0, 1 % NP40, 10 % glycerol, 1 mM phenylmethylsulfonylfluoride (PMSF), 10lg mL-1 aprotinin, 0.1 mM benzethonium chloride, and 0.5 mM sodium vanadate Homogenates were then centrifuged, supernatants were collected, and total protein concentration was determined according to the MicroBCA procedure (Pierce, IL, USA), using bovine serum albumin (BSA) as standard. Briefly, protein samples were separated by electrophoresis on a 10 and 12.5 % polyacrylamide gel (according to protein molecular weight), and electrotransferred to a PVDF membrane (Millipore, MA, USA). Non-specific binding sites were blocked in Tris-buffered saline (TBS), pH 7.6, containing 5 % non-fat dry milk. Membranes were rinsed in buffer (0.05 % Tween-20 in TBS) and then incubated with primary antibodies: anti-β-actin (1:2000), anti-KOR (1:1000), anti-NMDA (1:3000), anti-D1R (1:500) (Santa Cruz Biotechnology, Santa, Cruz, CA, USA) followed by anti-rabbit or anti-goat IgG horseradish peroxidase conjugate (1:40.000; Santa Cruz Biotechnology). After being rinsed with buffer, the immune complexes were visualized by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech Inc., NJ, USA) according to the manufacturer's instructions. The film signals were digitally scanned, and then quantified using ImageJ software. Actin was used as an internal control for Western blot such that data was standardized according to actin values.

Fatty acids profile in brain tissues

The fat was extracted from hippocampus and spinal cord using chloroform and methanol as described by Bligh and Dyer (1959) and used for determination of the fatty acid profile. To prevent lipid oxidation during and after extraction, 0.02% butyl hydroxy toluene was added to

the chloroform used. Fatty acid composition was determined by gas chromatography. Fat extracted from tissues and the interesterified fat were saponified in methanolic KOH solution and then esterified in methanolic H_2SO_4 solution (Hartmann and Lago1973). Methylated fatty acids were analyzed using an Agilent Technologies gas chromatograph (HP 6890N) equipped with a capillary column (DB-23 60 m x 0.25 mm x 0.25 μm) and flame ionization detector. The temperature of the injector port was set at 250°C and the carrier gas was nitrogen (0.6 mL/min). After injection (1 μL, split ratio 50:1), the oven temperature was hold at 150°C for 1 min, the it was increased to 240°C at 4°C/min and hold at this temperature for 12 min. Standard fatty acid methyl esters (37-component FAME Mix, C 22:5n3 and PUFA no. 2 from Sigma, Saint Louis, MO, USA and C 22:5n-6 from NuChek Prep. Inc., Elysian, MN, USA) were run under the same conditions and the subsequent retention times were used to identify the fatty acids. Fatty acids were expressed as percentage of the total fatty acids identified.

Statistical analysis

Two-way ANOVA followed by Newman−Keuls test was used (Software package Statistic 8.0 for Windows). All the data are expressed as means ±standard error of the mean (S.E.M.). Values of $p<0.05$ were considered statistically significant for all comparisons made. GraphPad Prism® (version 5.01) was used to create the figures.

Results

Development of morphine preference assessed in conditioned place preference (CPP)

Two-way ANOVA of CPP revealed a significant main effect of both supplementation and drug [$F(1,20)=7.2$, $p<0.05$; $F(1,20)=6.9$, $p<0.05$]. *Post-hoc* test revealed that SO supplemented animals and morphine conditioned showed increased time spent in the drug-conditioned place, while IF group showed no preference for neither sides of CPP (Fig. 2).

Anxiety-like symptoms evaluated in elevated plus maze (EPM) task

Two-way ANOVA of EPM revealed a significant main effect of supplementation, drug and supplementation x drug interaction on time spent in the open arms [$F(1,20)=9.73$, $p<0.01$; $F(1,20)=16.12$, $p<0.01$ and $F(1,20)=11.23$, $p<0.01$, respectively] and a significant effect of supplementation and drug on number of open arms entries [$F(1,20)=9.46$, $p<0.01$;

F(1,20)=5.84, p<0.05, respectively]. *Post-hoc* test showed that morphine withdrawal reduced the time spent (Fig. 3A) and the entries number in the open arms (Fig. 3B) of SO supplemented animals in relation to its vehicle. While IF group showed no differences in these parameters after morphine withdrawal, IF supplemented animals showed reduced anxiety index when compared to SO group (Fig. 3C).

Assessment of hyperalgesia with the hot plate test

A two-way ANOVA of hot plate test revealed a significant main effect of supplementation [F(1,20)=66.7, p<0.01]. *Post-hoc* test showed that IF supplemented animals showed lower latency to paw removal *per se*. During morphine withdrawal, SO supplemented animals showed lower latency response to paw removal in relation to its vehicle group, while IF group showed no difference (Fig. 4).

Influence of interesterified fat on immunoreactivityof D1R and NMDAR in hippocampus

Two-way ANOVA of D1R immunoreactivity revealed a significant main effect of drug [F(1,16)=9.95, p<0.01] in the hippocampus. *Post-hoc* test showed that morphine conditioning induced an increase in D1R immunoreactivity in SO group, while no difference was observed in IF group (Fig. 5A). Two-way ANOVA of NMDA immunoreactivity revealed a main effect of supplementation [F(1,16)=10.64, p<0.01] in the hippocampus. *Post-hoc* test showed increased NMDAR immunoreactivity morphine-induced in relation to vehicle in SO group (Fig. 5B).

Influence of interesterified fat on immunoreactivity of KOR in spinal cord

Two-way ANOVA of KOR immunoreactivity showed a significant main effect of supplementation [F(1,16)=68.13, p<0.00] in spinal cord. *Post-hoc* test showed that IF supplemented group increased KOR immunoreactivity, independently of morphine conditioning (Fig. 6A).Also, a negative linear correlation between KOR immunoreactivity and latency time (sec) on hot plate test (r^2=0.67, p=0.0000) was observed (Fig. 6B).

Fatty acids composition

One-way ANOVA of FA composition in hippocampus and spinal cord of rats supplemented with SO or IF showed no significant difference (data not shown).

Discussion

The aim of this study was to evaluate the influence of newest dietary fat, the interesteresfied fat (IF), when it was provided during early periods of life, through behavioral parameters and molecular markers associated to opioid drug conditioning. Thus, IF supplementation during gestation, lactation and postweaning (until PND 38) periods exerted alterations on morphine-induced hedonic and analgesic properties, affecting behaviors related to addiction. Our findings indicated that IF supplemented rats showed no preference for morphine, presenting no anxiety behaviors, which are commonly related to drug withdrawal, besides hyperalgesia *per se* and increased KOR immunoreativity in the spinal cord. Contrarily, SO supplemented animals, which was considered a control group, showed morphine-conditioned place preference (CPP) along with anxiety-like symptoms during drug withdrawal, as well as increased D1R and NMDAR immunoreactivity in the hippocampus.

The activation of opioid receptors, which are negatively coupled to Gi proteins, activates the dopaminergic system, leading to reward effects (Narita et al. 2001; Murakawa et al. 2004; Hirose et al. 2005; Ford et al. 2006). As shown by Lintas (2011), dopaminergic neurotransmission through the D1R is able to control acquisition of opioid reward memory, whose activation has been experimentally related to preference for morphine (Narita et al. 2004, 2005). In opposition, a blockade of D1R may prevent the opioid reward memory in the CPP model (Lintas et al. 2011; Beninger and Miller 1998). Studies have shown that D1R are co-located in the same neurons that D3R (Le Moine and Bloch 1996; Surmeier et al. 1996), and the repeated activation of D1R may regulate both D1R and D3R, suggesting an interaction between them (Bordet et al. 1997; Levavi-Sivan et al. 1998). Taken together, both D1R and D3R have been closely involved in drug addiction (Sokoloff et al. 2001; Newman et al. 2012; Song et al. 2013), while the involvement of D2R (Volkaw et al. 2001;Kei et al. 2015) and D4R (Rubinstein et al. 1997; Thanos et al. 2010) has shown contradictory evidences.

Instead to what was observed in SO, when IF-supplemented group was conditioned with morphine, hippocampal D1R immunoreactivity was not modified, suggesting that there was no activation of the dopaminergic pathway, which is necessary for the morphine rewarding effects. In fact, in the CPP paradigm exists a relationship between reward, mediated by increased DA content, and the learning process, when animals associate the drug hedonic effects to the environmental cues of the place where they were conditioned (Rolls and Xiang 2005).Thus, hippocampus is a major brain structure involved in spatial learning and memory (Bao et al. 2007), since evidences have shown that this brain area is centrally involved in the restoration

and distribution of information associated to drug addiction (Nestler et al. 2001), reward (White et al., 1996; Rezayof et al. 2003)as well as drug-seeking behavior (Vorel et al. 2001; Black et al. 2004; Yang et al. 2004).

In this same line of thinking, it is well known that the withdrawal of opioid drugs results in abstinence symptoms such as dysphoria, agitation, irritability and anxiety, which are present in both humans and experimental studies (Koob and Le Moal 2005; Hodgson et al. 2008; Zhang and Schulteis 2008). In this context, as observed in the EPM, morphine withdrawal of SO supplemented group was related to reduced number of entries and less time spent in the open arms of the apparatus, indicating higher anxiety degree. In addition, IF supplemented animals did not demonstrate development of anxiety during morphine withdrawal, as observed by lower anxiety index. Considering that IF supplemented animals showed no preference for morphine, indicating absence of addictive effects of the drug, they did not showed anxiogenic effects related to drug withdrawal.

Besides involvement of D1R, literature data have shown a relationship between mophine-induced NMDAR activation and reward mechanism (Beutler et al. 2011; Coleman et al. 2013; Sikora et al. 2016). Repeated administration of morphine was related to NMDAR activation (Mao et al. 2002), which exerts an important role in the opioid tolerance development (Lim et al. 2005; Sanchez-Blazquez et al. 2009). In fact, NMDAR antagonist is recognized by inhibits opioid tolerance developments (Manning et al. 1996). Following our understanding, we are showing by the first time that morphine conditioning in IF supplemented animals showed no changes in the NMDAR immunoreactivity, which was clearly increased in the SO supplemented group, suggesting that IF consumption is able to affect both glutamatergic and dopaminergic neurotransmission.

Especially important, one of mainly mechanisms underlying the development and pain maintenance is the activation of NMDAR (Yan et al. 2013).Activation of opioid receptors reduces the inhibitory GABAergic neurotransmission (Narita et al. 2005), which is involved in the negative control of pain, thus increasing the glutamatergic neurotransmission by activation of NMDA receptors (Basbaum et al. 2009). In this sense, opioid drugs remain in the therapeutic arsenal asdrugs of choice for relief deep pain, but unfortunately, these drugs also induce serious adverse effects, including hyperalgesia and analgesic tolerance (Mao et al. 1995), impairing the pain treatment. In our study, morphine withdrawal of SO group was related to both increased hippocampal NMDAR immunoreactivity, and reduced latency to paw removal on the hot plate test, inferring that these animals had a higher sensibility to the thermal stimulus presented.

These findings suggest development of hyperalgesia to opioid, as described before (Hutchinson et al. 2011;Xin et al. 2012). According to our data, NMDAR play an important role in both opioid-induced tolerance and hyperalgesia development (Mao et al. 1994; Trujillo et al. 1991), and are highly involved in pain states and addiction (Lau and Zukin 2007), as observed in SO supplemented group that was exposed to morphine conditioning. Surprisingly, chronic IF consumption during early life periods did not change NMDAR immunoreactivity, presenting no hyperalgesia to opioid. However, this experimental group presented hyperalgesia *per se* to heat stimuli, indicating an increased thermal sensibility, which was negatively correlated with KOR immunoreactivity in the spinal cord, independently of morphine conditioning. Indeed, while kappa opioid system can be involved in behavioral responses related to anxiety-like symptoms (Sun et al. 2010; 2014; Zádor et al. 2015), KOR are intensely expressed in the spinal cord, which is a relay center of sensory signals including pain (Wang et al. 2009).

Furthermore, during a pain state occurs the activation of immune cells, which are responsible for the synthesis and secretion of opioid peptides endogenous, such as dynorphins, thus activating KOR (Labuz et al. 2016). Activation of KOR is consistent with the activation of dynorphins system (Vanderah et al. 2000). Our current experimental protocol did not include binding studies involving dynorphins and KOR, but we think that an increased KOR activation may has occurred in the IF group, since these animals showed no morphine preference. In fact, supra activation of KOR is able to regulates negatively the dopaminergic system that is mediated by opioid system (Chefer et al. 2005; Narita et al.2005), thus decreasing morphine reward effects. Based on this, we hypothesized that the thermal hyperalgesia consequent to the chronic consumption of IF throughout early life periods may be related to release of opioid peptides endogenous such as dinorphins, which are able to activate KOR, and negatively modulate reward- and anxiety-like symptoms induced by morphine, as demonstrated in the current study.

Of particular importance, previous studies from our laboratory have shown a relationship between increased preference for addictive drugs and *trans* fat derivatives incorporation into neural membrane phospholipids (Kuhn et al. 2015; Trevizol et al. 2015; Dias et al. 2015), affecting both brain derived neurotrophic factor (BDNF) and D1R immunoreactivity (Kuhn et al. 2015). IF is composed by a rearrangement of different types of FA that are found in the dietary. In this sense, it is difficult to detect a specific IF molecule, since our current analytic method is able to detect only the fatty acids in IF composition, but no its metabolites, unlike hydrogenated vegetable fat, which is mainly composed by fatty acids

presenting the *trans* conformation. Despite the difference in the FA composition of supplementations, our current study showed no changes in the FA composition in both hippocampus and spinal cord of IF supplemented animals. In fact, while FA incorporation in the brain membranes is selective, diets with low amount of essential FA, such as docosahexae noicacid (DHA) and arachidonicacid (AA), are mainly incorporated in CNS (Tu et al. 2013). The IF fatty acids analysis showed that it has a high percentage of saturated FA associated with reduced polyunsaturated FA content when compared to SO, thus favoring the increased n-6/-3 ratio at levels above those acceptable for a healthy diet.

As the use of IF in processed foods is recent, no studies about the influence of its chronic consumption were developed so far, and we do not have a mechanism of action to explain both behavioral and molecular outcomes that were observed in IF supplemented animals. Regarding this, we can propose some hypotheses that deserve to be considered and discussed: i) FA derived from IF could act directly on G-protein receptor 40 (GPR40), which may be physiologically affected by DHA, AA and others FA (Briscoe et al. 2003; Hirasawa et al. 2005; Nakamoto et al. 2012; 2013). Of particular importance for our findings, studies have shown that GPR40 activation is able to increase β-endorphins release, contributing to alleviate pain conditions (Nakamoto et al. 2013). We hypothesized that IF derivatives may differently affect GPR40R, modifying β-endorphins release, and then modifying pain sensibility, as observed in our current study; ii) dietary DHA is favorably incorporated into neural membrane phospholipids thus minimizing the phospholipase A2 activity (Kim et al. 1999). Such property disfavors pro-inflammatory precursors generation, decreasing neuroinflammation processes (Massaro et al. 2008). Unfortunately, IF metabolites are unknown so far, as well as their properties on the neural membrane.

Currently, most developed countries, IF fat is replacing *trans* fat in food processing, once *trans* fat is being reduced or prohibited (L'Abbè et al. 2009; Reming et al. 2010; Gagliardi et al. 2013). The replacement of *trans* fat by IF can be equally or even more harmful to health, and regarding this, health authorities should be alert to potential damage apparently silent resulting from chronic use of synthetic fats. Significant changes on neurotransmission can be reflected in an increased susceptibility to development of neuropsychiatric diseases (Teixeira et al. 2011; Trevizol et al. 2011; 2014; Pase et al. 2013; 2015, Dias et al. 2015) since dietary fatty acids may be reflected on behavioral, neurochemical and brain molecular changes as shown in this study.

Taken together, we propose that chronic consumption of foods rich in IF can affect neurotransmission resulting in loss of the rewarding effects induced by morphine administration in rats. This study contributes to a related warning to public health, since it is showing the harmful influence of IF on dopaminergic and glutamatergic neurotransmission, which are confirmed by molecular markers, so affecting rewards system related to opioid activation.

To the best of the author's knowledge, no previous publications concerning the role of IF on the opioid system were available at this time. Further studies are needed to determine a possible link among chronic consumption of IF, drug addiction and loss in reward responses related to morphine administration.

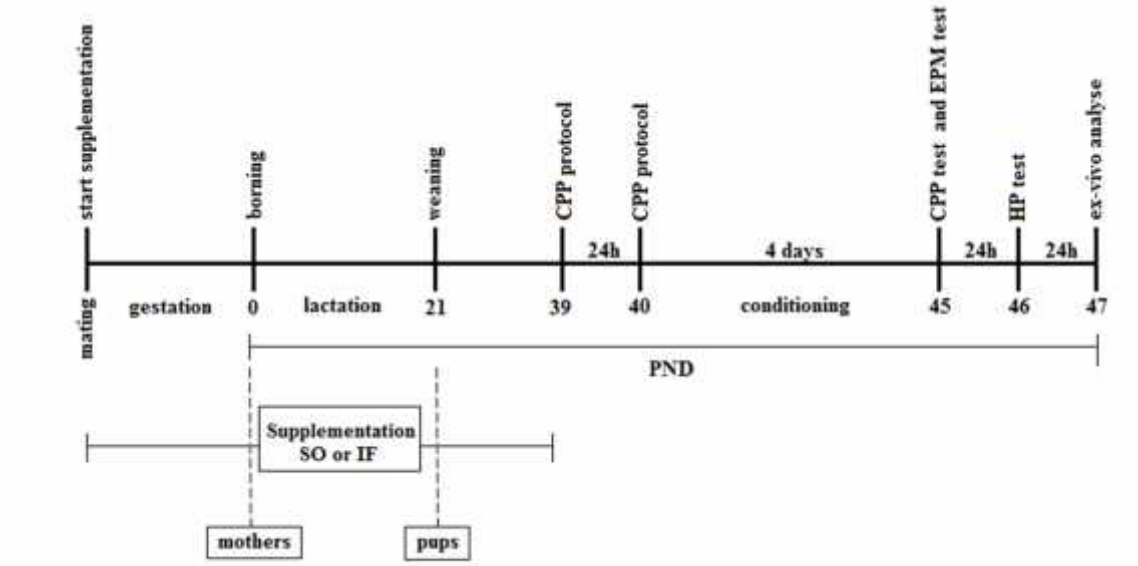

Figure 1: Experimental procedure: During gestation and lactation, dams were orally supplemented with SO and IF, which were maintained for the pups since post-weaning until adolescence, when female pups were conditioned with morphine in CPP paradigm. On PND45 (adolescence period), preference for morphine (4mg/kg, i.p.; for 4 days in CPP), anxiety-like symptoms related to drug withdrawal (EPM) and thermal sensibility were assessed. Abbreviations: SO: soybean oil; IF: interesterified fat; PND: posnatal day CPP: conditioned place preference; EPM: elevated plus maze; HP: hot plate.

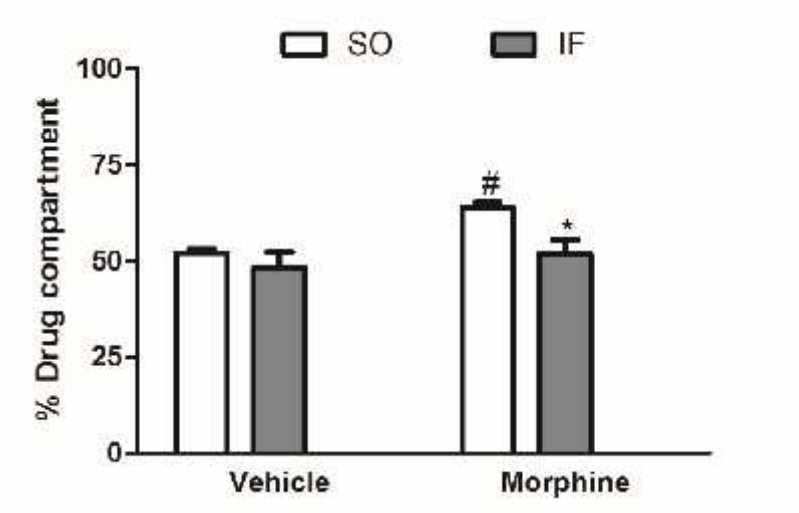

Figure 2: Influence of SO / IF supplementation during pregnancy, lactation and post-weaning on morphine- CPP (conditioned place preference). Data are expressed as mean ±S.E.M. #indicates significant difference between treatments in the same supplementation (p<0.05); *indicates significant difference between supplementations in the same treatment (p<0.05). Abbreviations: SO: soybean oil; IF: interesterified fat.

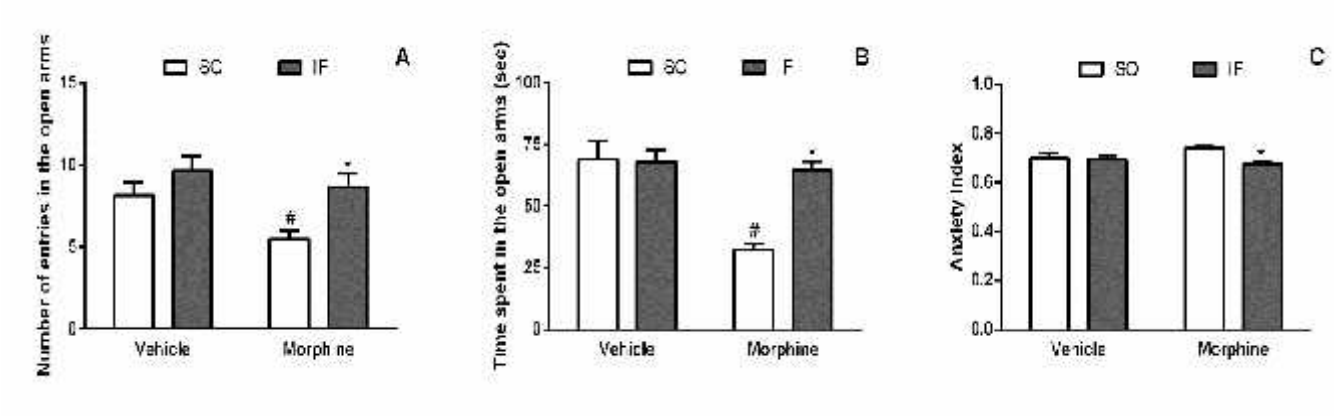

Figure 3: Influence of SO / IF supplementation during pregnancy, lactation and post-weaning on anxiety symptoms in elevated plus maze (EPM) task. (A) Entries number in the open arms; (B) Time spent in the open arms; (C) Anxiety index. Data are expressed as mean ±S.E.M. #indicates significant difference between treatments in the same supplementation; $p<0.05$ *indicates significant difference between supplementations in the same treatment, $p<0.05$.Abbreviations: SO: soybean oil; IF: interesterified fat.

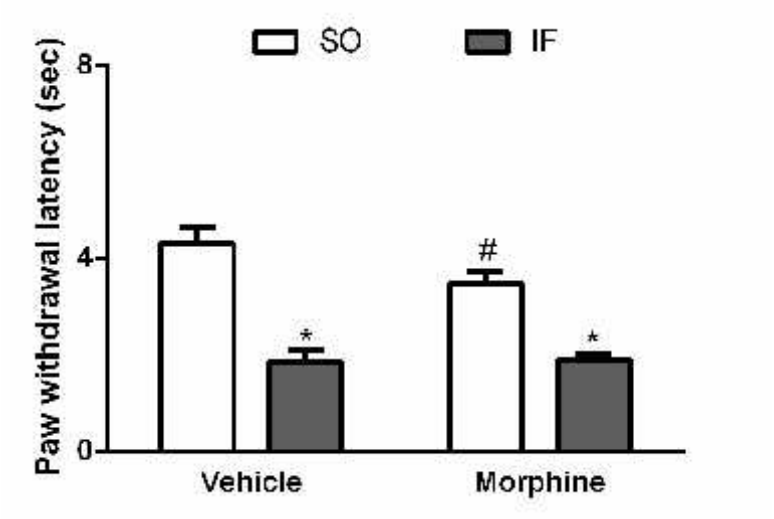

Figure 4: Influence of SO / IF supplementation during pregnancy, lactation and post-weaning on response latency on thermal stimuli in the hot plate (HP) test (55°C). Data are expressed as mean ± S.E.M. #indicates significant difference between treatments in the same supplementation; $p<0.05$ *indicates significant difference between supplementations in the same treatment, $p<0.05$. Abbreviations: SO: soybean oil; IF: interesterified fat.

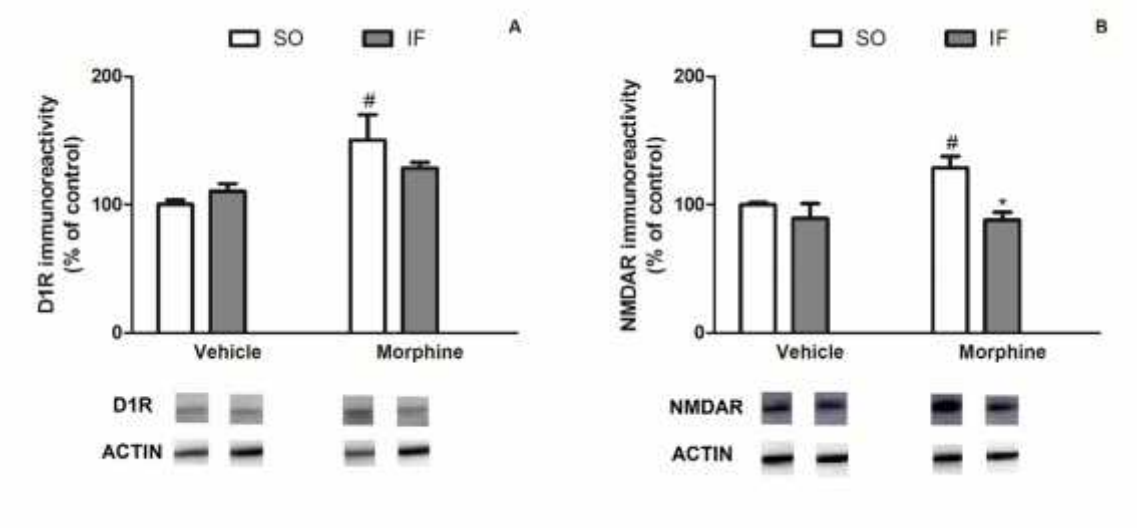

Figure 5: Influence of SO / IF supplementation during pregnancy, lactation and post-weaning on (A) D1R and (B) NMDAR immunoreactivityin hippocampus; Data are expressed as mean ±S.E.M. #indicates significant difference between treatments in the same supplementation; $p<0.05$ *indicates significant difference between supplementations in the same treatment, $p<0.05$. Abbreviations: Abbreviations: SO: soybean oil; IF: interesterified fat; D1R: Dopamine 1 receptor; NMDAR: N-methyl-D-aspartate receptor.

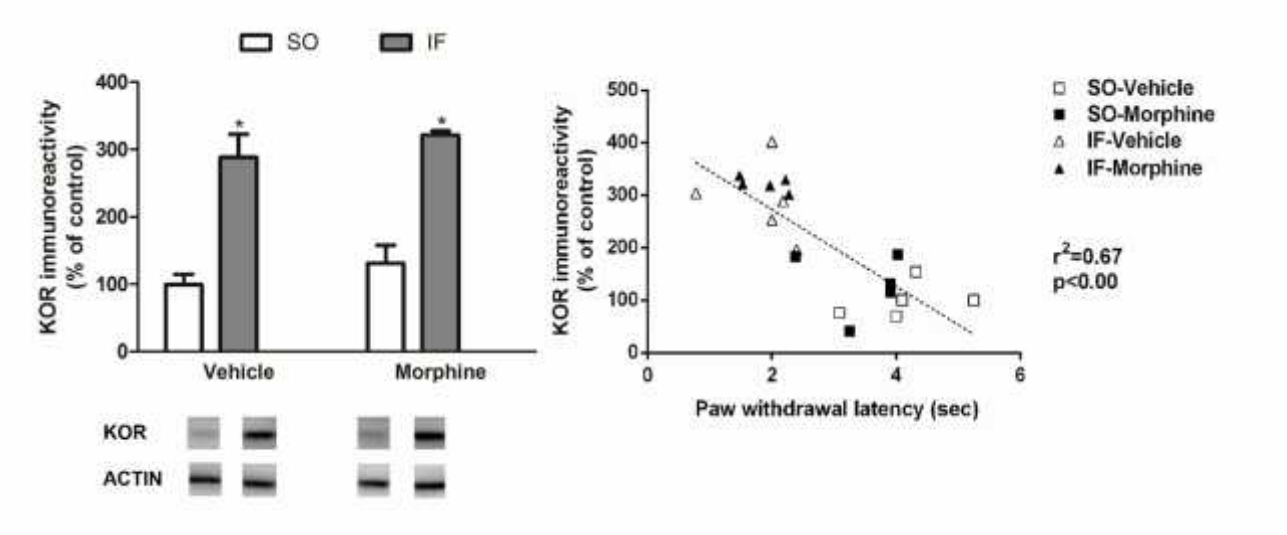

Figure 6: Influence of SO / IF supplementation during pregnancy, lactation and post-weaning on (A) KOR immunoreactivity in spinal cord (B) Correlation between KOR immunoreactivity and latency on hot plate test. Data are expressed as mean ±S.E.M. *indicates significant difference between supplementations in the same treatment, p<0,05. Abbreviations: SO: soybean oil; IF: interesterified fat; KOR: Kappa opioid receptor.

Table 1. Fatty acid composition (% of total identified FA) of the chow and the different dietary supplementation

Fatty acids	Chow	SO	IF
Σ SFA	25.50	18.00	55.30
Σ MUFA	34.40	26.00	33.70
Σ TFA	0.50	0.15	0.58
Σ n-6	37.30	50.30	10.10
Σ n-3	2.90	5.50	0.30
n-6/n-3	13.00	9.20	31.6

SFA: saturated fatty acids; MUFA: Monounsaturated fatty acids; TFA: *trans* fatty acids; SO: soybean oil; IF: interesterified fat.

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5. CONCLUSÕES

A partir dos resultados experimentais obtidos no presente estudo, pode-se chegar as seguintes conclusões:

- ✓ A suplementação de GI nos períodos iniciais do desenvolvimento influenciou negativamente a função das vias neurais opióides envolvidas no sistema de recompensa;

- ✓ A suplementação de GI prejudicou a ativação dopaminérgica e glutamatérgica induzida pelo sistema opioide, a qual foi observada no grupo suplementado com óleo de soja (controle isocalórico);

- ✓ O grupo GI apresentou hiperalgesia quando exposto a placa quente, indicando uma hipofunção do sistema opioide;

- ✓ Os animais suplementados com GI apresentaram maior imunorreatividade dos receptores kappa opioides, a qual foi negativamente correlacionada ao desenvolvimento de hiperalgesia.

6. PERSPECTIVAS

Com base nos resultados obtidos aqui, estudos mais aprofundados fazem-se necessários com a finalidade de investigar os mecanismos envolvidos nas respostas comportamentais e moleculares decorrentes do consumo de gordura interesterificada. Além disso, será conduzido uma estudo epigenético para avaliar os efeitos do consumo desta gordura sobre a prole subseqüente ou de 2ª geração, a nível comportamental bem como sobre os sistemas e vias de sinalização envolvidos.

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ANEXO:

Carta de aprovação do projeto na Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria.

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "Influência de tipos diferentes de gorduras em relação a parâmetros comportamentais e bioquímicos e sua interação com fármaco benzodiazepínico em ratos", protocolada sob o CEUA nº 1391090616, sob a responsabilidade de **Marilise Escobar Bürger** - que envolve a procução, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 5.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 08/09/2016.

We certify that the proposal "Influence of the types of fats with respect to behavioral and biochemical parameters and their interaction with benzodiazepine drug in rats", utilizing 48 Heterogenics rats (28 males and 20 females) protocol number CEUA 1391090616, under the responsibility of **Marilise Escobar Bürger** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientifc research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 09/08/2016.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 08/2016 a 08/2020 Área: Farmacologia

Origem:	Biotério Central UFSM						
Espécie:	Ratos heterogênicos	sexo:	Machos	idade:	60 a 60 dias	N:	28
Linhagem:	Wistar			Peso:	300 a 300 g		
Origem:	Biotério Central UFSM						
Espécie:	Ratos heterogênicos	sexo:	Fêmeas	idade:	60 a 60 dias	N:	20
Linhagem:	Wistar			Peso:	300 a 300 g		

Resumo: A gordura interesterificada (GI) vem sendo utilizada, em alimentos processados, como substituta da gorcura vegetal hidrogenada, rica em ácidos graxos trans (AGT). Apesar da GI não apresentar AGT em sua estrutura,estudos mostram que ela desencadeia os mesmos problemas cardiovasculares e risco aumentado de desenvolver diabetes relacionados com o consumo desse ácido graxo. Porém ainda não existem estudos sobre o efeito do consumo da GI sobre o desenvolvimento de estresse oxidativo e alterações neurais relacionadas com doenças neuropsiquiátricas tal como a ansiedade como é observado pelo consumo de AGT. Através do modelo animal de estresse crônico e moderado, propomos avaliar comparativamente a influência da suplementação diária de óleo de soja (controle isocalórico), gordura vegetal hidrogenada (rica em AGT) e gordura interesterificada, durante a gestação, lactação até a idade adulta de ratos Wistar e a exposição ao diazepam, sobre parâmetros comportamentais de ansiedade.Parâmetros de estresse oxidativo serão avaliados a partir da determinação de carbonilação de proteínas e níveis de malondialdeído em hipocampo, córtex pré-frontal e plasma e nos mesmos tecidos será avaliado a densidade do receptor GABAA,será realizado também a quantificação dos níveis de corticosterona será realizada no plasma.

Local do experimento: Laboratório de Farmacologia e Toxicologia- FARMATOX- Prédio 21, Sala 5220-UFSM

Santa Maria, 13 de setembro de 2016

Profa. Dra. Daniela Bitencourt Rosa Leal
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Denis Broock Rosemberg
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Avenida Roraima, 1000, Reitoria, 2º andar - CEP97105-900 Santa Maria, RS - tel: 55 (55) 3220-9362 / fax
Horário de atendimento: das 8:30 às 11h e 14h às 17hs : e-mail: ceua.unsm@gmail.com
CEUA/UFSM 1391090616