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**Diéssica Padilha Dalenogare**

# EFEITO DO ÁCIDO CAFEICO E ÁCIDO CAFEICO FENETIL ÉSTER EM MODELO DE INFLAMAÇÃO INDUZIDA POR LIPOPOLISSACARÍDEO

Santa Maria, RS, Brasil, 2016

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Área de concentração em Enzimologia Toxicológica, da Universidade Federal de Santa Maria (UFSM,RS), como requisito parcial para a obtenção do grau de **Mestre em Ciências Biológicas: Bioquímica Toxicológica**

Orientadora: Prof.ª Dr.ª Vera Maria Melchiors Morsch

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**Vera Maria Melchiors Morsch, Dra(UFSM)**
(Presidente/Orientadora)

**Daniela Bitencourt Rosa Leal, Dra(UFSM)**

**Jamile Fabbrin Gonçalves, Dra(IFFar)**

Santa Maria,RS
2016

## DEDICATÓRIA

A minha mãe, pelo exemplo de amor e dedicação.

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“Não te rendas que a vida é isso,
Continuar a viagem, perseguir teus sonhos,
Destravar o tempo, correr os escombros,
E destapar o céu.”.
Mário Benedetti

## RESUMO

### EFEITO DO ÁCIDO CAFEICO E ÁCIDO CAFEICO FENETIL ÉSTER EM MODELO DE INFLAMAÇÃO INDUZIDA POR LIPOPOLISSACARÍDEO

Autor: Diéssica Padilha Dalenogare

Orientadora: Vera Maria Melchiors Morsch

A inflamação periférica é capaz de causar alterações no sistema nervoso central (SNC) e levar a progressão de várias doenças neurodegenerativas. Durante o processo de neuroinflamação ocorre a ativação de células imunes do SNC e a infiltração de células imunes periféricas que acabam por liberar elevados níveis de citocinas, levando ao estresse oxidativo. O lipopolissacarídeo (LPS) é um componente biologicamente ativo da membrana de bactérias gram-negativas e é responsável pela sua toxicidade sendo capaz de estimular o sistema imunológico. Esta ativação leva ao aumento da expressão de citocinas pró-inflamatórias, tais como a interleucina-1β, Interleucina-6, fator de necrose tumoral alfa (TNF-α) e produção de espécies reativas. Porém, existe um complexo sistema de proteção da célula composto de enzimas antioxidantes, tais como o sistema antioxidante de glutationa e outros diversos fatores antioxidantes não enzimáticos. Os compostos fenólicos apresentam diversas funções, dentre elas a função antioxidante e anti-inflamatória que podem modular e prevenir os danos causados pelo estresse oxidativo. Desta forma, investigou-se o pré-tratamento com ácido cafeico (AC) e ácido cafeico fenetil éster (ACFE) previniu alterações em parâmetros comportamentais e de estresse oxidativo em córtex de camundongos expostos a um modelo de inflamação sistêmica induzida por LPS. Os camundongos Swiss foram divididos em seis grupos: controle (óleo de milho), controle/AC 50 mg/kg, controle/ACFE 30 mg/kg, LPS 250µg/kg (diluído em salina), LPS/AC 50 mg/kg, LPS/ACFE 30mg/kg pré-tratados via oral por gavagem durante 30 dias, onde ambos os compostos AC e ACFE foram diluídos em óleo de milho. Após este período foram anestesiados, eutanasiados e o córtex cerebral retirado para análises. De acordo com os resultados pode-se observar a prevenção da perda de memória somente nos animais do grupo LPS/ACFE 30mg/kg, sendo que os demais grupos não apresentaram diferença significativa na atividade locomotora e de memória. Em relação aos parâmetros de estresse oxidativo ficou demonstrado que o LPS foi capaz de aumentar os níveis de espécies reativas de oxigênio, a carbonilação proteica e os níveis de nitrito e de nitrato. Já os compostos AC e ACFE demonstraram efeito protetor nos parâmetros de estresse oxidativo desenvolvido pela injeção de LPS em amostras de córtex apresentando uma diminuição dos níveis de nitrito e nitrato, da carbonilação protéica e dos níveis de espécies reativas. Além disso, o AC e o ACFE apresentaram também efeito protetor na manutenção do sistema glutationa. Desta forma, pode-se indicar que ambos os compostos fenólicos, AC e ACFE exercem as funções antioxidantes frente aos danos oxidativos desenvolvidos pela injeção de LPS no SNC.



**Palavras-chaves:** Estresse oxidativo. Compostos fenólicos.sistema nervoso central. Antioxidantes

## ABSTRACT

### EFFECTS OF CAFFEIC ACID AND CAFFEIC ACID PHENETYL ESTER ON INFLAMMATION LIPOPOLISACHARYDE-INDUCED

Author: Diéssica Padilha Dalenogare
Advisor: Vera Maria Melchiors Morsch

Peripheral inflammation is able to cause alterations in central nervous system (CNS) and lead to progression of neurodegenerative diseases. During neuroinflammatory process the activation of immune cells from the CNS and infiltration of peripheral immune cells occurs, which eventually release high levels of cytokines leading to oxidative stress. Lipopolysaccharide (LPS) is a biological active component of the membrane of gram-negative bacteria and is responsible for their toxicity being able to stimulate the immune system. This activation leads to increased expression of pro inflammatory cytokines such as interleukin 1β, IL-6, tumor necrosis factor (TNF-α) and production of reactive species. However, a large cell protection system is present composed of antioxidant enzymes such as glutathione antioxidant system and many other nonenzymatic antioxidants factors. Phenolic compounds have several functions, including antioxidant and anti-inflammatory function that can modulate and prevent damage caused by oxidative stress. Thus, it is intended to investigate the effect of treatment with caffeic acid (CA) and caffeic acid phenethyl ester (CAPE) on anti-oxidative stress and behavioral parameters in cortex of mice exposed to a systemic inflammatory model induced by LPS. The Swiss mice were divided into six groups: control (corn oil), control / CA 50 mg / kg, control/ CAPE 30 mg/ kg LPS 250μg / kg, LPS / CA 50 mg / kg LPS / CAPE 30mg / kg pre-treated orally by gavage during 30 days, both compounds were diluted with corn oil. After this period they were anesthetized, euthanized and the cerebral cortex removed for analysis. According to the results, we can observe the prevention of memory loss only in the animals of group LPS/CAPE 30mg / kg, and the other groups showed no significant difference in locomotor activity and memory. Regarding the oxidative stress parameters it demonstrated that LPS was able to increase the levels of reactive oxygen species, protein carbonyls and the levels of nitrite and nitrate. Already the AC and ACFE compounds showed protective effect on oxidative stress parameters developed by the injection of LPS in cortex samples, it was showing decreased levels of nitrite and nitrate, the protein carbonyls and levels of reactive species. In addition, AC and ACFE also showed a protective effect in maintaining glutathione system. Thus, it can be stated that both phenolic compounds, AC and ACFE exert antioxidant functions to forward oxidative damage developed by LPS injection in the CNS.



**Key words:** Oxidative stress. Phenolic compounds. Central nervous system. Antioxidant

## LISTA DE ILUSTRAÇÕES

### REFERENCIAL TEÓRICO



### MANUSCRITO

## LISTA DE ABREVIATURAS E SIGLAS

AC – Ácido cafeico

ACFE – Ácido cafeico fenetil éster

BHE – Barreira hematoencefálica

GSH – Glutationa

GPx – Glutationa peroxidase

GR – Glutationa redutase

GSSG – Glutationa oxidada

IL-1β– Interleucina 1β

IL-6 –Interleucina 6

IL-10 – Interleucina 10

IL-11– Interleucina 11

LPS – Lipopolissacarídeo

NF-κB – Fator de necrose κB

NO – Óxido nítrico

SNC –Sistema nervoso central

## LISTA DE ANEXOS

**SUMÁRIO**

## 1 INTRODUÇÃO

A inflamação periférica é capaz de causar alterações como deterioração sináptica, morte neuronal e exacerbação dos processos envolvidos nas doenças neurodegenerativas no sistema nervoso central (SNC) e levar a progressão de várias doenças neurodegenerativas (LYMAN et al., 2014; PERRY et al., 2010). No processo inflamatório do SNC, também conhecido como neuroinflamação, ocorre a ativação de células imunes do cérebro e a infiltração de células imunes periféricas que acabam por liberar elevados níveis de citocinas levando ao estresse oxidativo (HOOGLAND et al 2015;. AGOSTINHO et al 2010). Como consequência disto temos uma disfunção neuronal e, consequentemente, alterações na função cognitiva (DANTZER et al. 2008). O aumento dos níveis de citocinas pró-inflamatórias e de marcadores de oxidação sugere uma associação com a diminuição da memória relacionada às doenças neurodegenerativas, como a doença de Alzheimer (GUERREIRO et al., 2007).

O lipopolissacarídeo (LPS) é um componente biologicamente ativo da membrana de bactérias gram-negativas sendo capaz de estimular o sistema imunológico (LU et al. 2008). O reconhecimento de agentes patogênicos pelo sistema imune tem vários receptores específicos, tais como receptores Toll-like, do inglês Toll-like receptors (TLRs). A hiperestimulação dos TLRs culmina no aumento da transcrição do fator nuclear kappa B (NF-κB), levando ao aumento da expressão de citocinas pró-inflamatórias, tais como a interleucina 1β, IL-6, fator de necrose tumoral alfa (TNF-α ) e produção de espécies reativas. Além disso, esses eventos deletérios resultam em perda de neurônios do hipocampo e prejudicam a aprendizagem e formação da memória (CARVALHO et al 2016;. FRUHAUF et al 2015).

A inflamação sistêmica induzida por LPS envolve diversos mecanismos moleculares da inflamação e danos celulares que produzem espécies de oxigênio, como o óxido nítrico (NO), superóxido ($O_2^-$) ou peroxinitrito ($ONOO^-$) (KUMAR et al 2014;. KAWANO et al 2007). Esses danos levam a diminuição da função mitocondrial (CHOUMAR et al . 2011), sendo o sistema antioxidante da glutationa essencial na detoxificação dos radicais livres gerados durante a ativação do sistema imune. Deste modo a glutationa(GSH) atua como um co-factor para as enzimas antioxidantes glutationa peroxidase (GPx) e glutationa-S-transferase (GST). Além disso, o desbalanço do sistema de GSH tem sido relacionado com o estresse oxidativo que ocorre em doenças neurológicas, tais como a esquizofrenia (DO et al., 2000), a doença de Alzheimer

(BRAIDY et al. 2015) a epilepsia (MUELLER et al., 2001) e doença de Huntington (RIBEIRO et al. 2012).

O estresse oxidativo e liberação de mediadores inflamatórios têm sido relatados como fatores importantes nos eventos dos processos patológicos do SNC (CARVALHO et al. 2016). Ao mesmo tempo, os agentes antioxidantes que atuam sobre a redução destes fatores são capazes de controlar efeitos nocivos da neuroinflamação. Assim, a descoberta de agentes que modulam este processo pode promover uma melhoria no prognóstico de processos patológicos associados com neuroinflamação, tais como a progressão de doenças neurodegenerativas (ALLAN & ROTHWELL 2001, 2003).

O ácido cafeico (AC) é um composto fenólico que é encontrado em frutas, café, azeite e em algumas ervas medicinais. A maioria dos derivados de CA existe na forma de ésteres, tais como o ácido cafeico fenetil éster (ACFE), um componente bioativo encontrado na própolis produzida por abelhas (BANSKOTA et al., 2001). Ambos AC e ACFE apresentam uma grande variedade de atividades biológicas, incluindo funções anti-inflamatória, antioxidante e imunomoduladoras (DESHMUKH et al 2016;. TSAI et al 2015;.. DOS SANTOS et al 2014; CUNHA et al., 2004). Estudos farmacológicos recentes têm mostrado que o AC exerce um efeito protetor contra danos oxidativos induzidos por peróxido de hidrogênio ($H_20_2$) no cérebro, e evita alterações comportamentais e bioquímicas em modelo de indução da doença de Alzheimer em ratos (DESHMUKH et a. 2016). Além disso, ACFE mostrou também efeitos protetores na geração de radicais livres e na neurotoxicidade induzida por ácido 3-nitropropiônico, um modelo de indução da doença de Huntington (DESHMUKH et ai. 2016). Tanto o AC quanto o ACFE inibem a ativação da transcrição do NF-κβ, inibindo a produção de prostaglandinas e cicloxigenases, o que lhe confere atividades anti-inflamatórias e imunomoduladoras (KANG et al 2009;. BOSE et al 2009;.. LEE et al 2004).

Neste contexto, considerando que a neuroinflamação está relacionada com os processos patológicos de doenças neurodegenerativas é de grande interesse a procura por agentes terapêuticos capazes de diminuir o estresse oxidativo devido a ativação do sistema imune.

## 1.1 OBJETIVOS

### 1.1.1 Objetivo geral

Avaliar o potencial terapêutico dos compostos fenólicos ácido cafeico e ácido cafeico fenetil éster sobre parâmetros comportamentais e de estresse oxidativo em camundongos expostos a um modelo de inflamação sistêmica.

### 1.1.2 Objetivos específicos

Em um modelo experimental de inflamação sistêmica induzido por LPS em camundongos, teremos como objetivo:

- Avaliar o efeito protetor do AC e ACFE em parâmetros comportamentais relativos à atividade locomotora e à memória;
-Investigar o efeito antioxidante do AC e ACFE em parâmetros de estresse oxidativo em amostra de córtex;
- Avaliar se os compostos AC e ACFE são capazes de alterar a atividade do sistema antioxidante de glutationa em córtex.

## 2 REFERENCIAL TEÓRICO

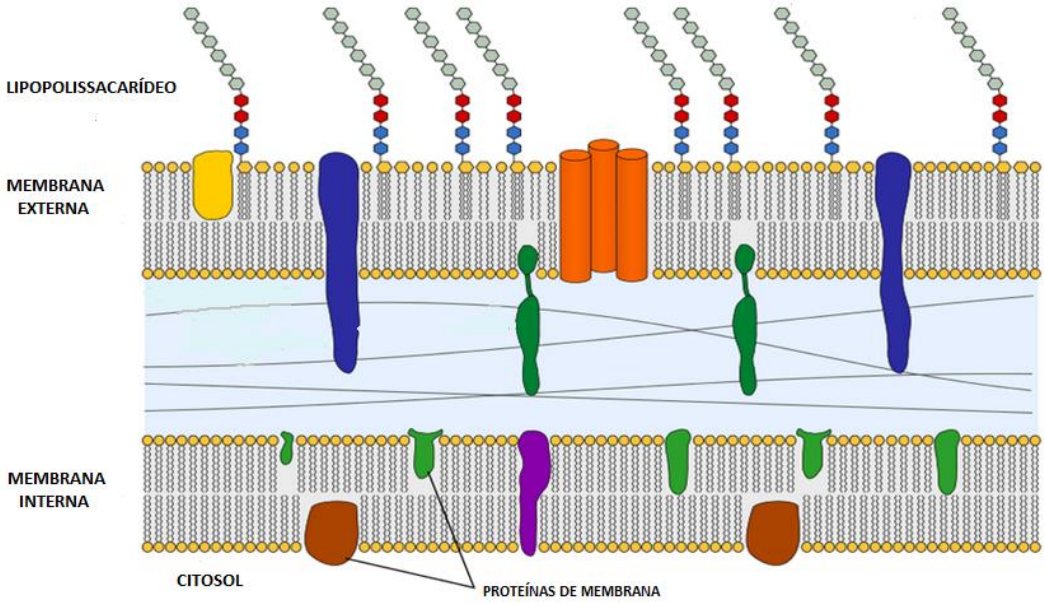
Neuroinflamação é o termo usado para descrever a grande extensão da resposta imunológica no sistema nervoso central (SNC) Esta resposta inflamatória resulta em deterioração sináptica, morte neuronal e exacerbação dos processos envolvidos nas doenças neurodegenerativas (LYMAN et al., 2014). Porém, para que ocorra o processo de neuroinflamação é necessário, primeiramente, que ocorra uma disfunção da barreira hematoencefálica (BHE) sendo um elemento-chave da progressão de várias doenças do SNC (CUNNINGHAM et al.,2009; KITAZAWA et al., 2005; MICHEAU & TSCHOPP, 2003).

A BHE tem como principal característica a impermeabilidade, apresentando componentes que dificultam a penetração das moléculas. Esta propriedade é baseada na existência de uma permeabilidade muito restrita do endotélio permitindo a passagem somente de moléculas como água, gases como oxigênio e o dióxido de carbono, e algumas moléculas lipossolúveis muito pequenas (BANKS, 2009). Há canais específicos para a passagem de moléculas essenciais para o metabolismo do cérebro, tais como íons, glicose e aminoácidos. Deste modo a BHE se torna altamente seletiva, mas pode ser incapaz de impedir a passagem de algumas toxinas e agentes terapêuticos da corrente sanguínea para o cérebro. Além disso, a BHE pode responder a estímulos periféricos secretando citocinas, prostaglandinas e oxido nítrico exercendo uma função neuroimunológica (BENGLEY, 2003; BANKS & ERICKSON, 2010).

Além da BHE, a micróglia, tipo celular da glia, ajuda na manutenção da permeabilidade seletiva fazendo parte do sistema imune do SNC. Dentre as funções desempenhadas por essas células temos a manutenção da homeostase do tecido cerebral podendo ser ativada caso ocorra alguma lesão ou injuria. Essas células são consideradas a primeira linha de defesa contra os agentes invasores, e através de interações com neurônios podem detectar mudanças críticas na atividade neuronal. Além disso, as células microgliais são capazes de fazer a remoção de células mortas ou danificadas, sendo considerados macrófagos específicos do SNC (HANISCH e KETTENMANN, 2007; RODRIGUES et al, 2014). Em resposta a moléculas de sinalização de inflamação aguda, a micróglia passa a exercer, portanto um estado ativo de fagocitose liberando mediadores pró-inflamatórios. As células microgliais ativadas podem liberar quantidades de citocinas e moléculas neurotóxicas que contribuem para neurodegeneração à longo prazo (RODRIGUES et al., 2014).

Diversos estudos mostram o modelo de indução de inflamação sistêmica por lipopolissacarídeo (LPS) como capaz de ativar a micróglia (HU et al., 2012; MANDER & BROWN, 2005; MONJE et al.; 2003). O LPS é uma endotoxina e componente lipídico da membrana externa das bactérias gram-negativas (RHEE, 2014). A liberação de moléculas de LPS através da parede bacteriana expõe a sua porção tóxica, o lipídio A, gerando uma resposta inflamatória do sistema imunológico.

Figura 1- Estrutura do lipopolissacarídeo




Adaptado de RHEE et al., 2014

O conceito de que o LPS é o principal fator de virulência de bactérias gram-negativas é baseado em estudos que mostram que o LPS purificado ou o lipídeo A sintetizado quimicamente são capazes de reproduzir em humanos e outros animais algumas manifestações semelhantes às induzidas por uma infecção na presença de bactérias (HEUMANN & ROGER, 2002). Durante a resposta inflamatória sistêmica são liberadas citocinas, as proteínas sinalizadoras que medeiam a neuroinflamação, como por exemplo, a Interleucina (IL)-6 e Fator de necrose tumoral (TNF)-α que fazem parte da resposta imune humoral (AL NIMER et al., 2011).

Também fazem parte da resposta humoral os chamado receptores Toll-like, do inglês Toll-like receptors (TLRs), que são considerados proteínas importantes na transdução de sinal no sistema imune e na resposta inflamatória, sendo ativados em caso de detecção de agentes

patológicos iniciando, assim, a cascata de sinalização (BADSHAH et al., 2016). O receptor TLR-4 é de particular importância uma vez que pode ser ativado na presença de LPS, liberando TNF-α e IL-1β. Por serem os receptores-chave na sinalização pró-inflamatória os astrócitos e a micróglia expressam uma enorme quantidade de receptores TLR-4, que ativam essas células e iniciam a reação neuroinflamatória (PARK & LEE, 2013). As citocinas TNF-α e IL-1β fazem parte do grupo de citocinas pró-inflamatórias, sendo que a IL-4, IL-10, IL-11 e IL-6 têm ação anti-inflamatória (OPAL & DEPALO, 2000). Dependendo da patologia há diferentes sinais moleculares, sendo que, quando há a secreção excessiva de citocinas pró-inflamatórias através dos astrócitos, pode resultar em uma ativação patológica e reações de estresse oxidativo.

O estresse oxidativo refere-se a uma condição em que a produção de espécies reativas supera a capacidade de defesa antioxidante celular do organismo. Várias evidências mostram a ligação do estresse oxidativo e nitrosativo desempenhando um papel prejudicial em patologias neurodegenerativas como a Doença de Alzheimer (DA) e a Doença de Parkinson (BUTTERFIELD et. al , 2010; BHARATH et al., 2002). Os radicais livres como o ânion superóxido, radical peroxila e radical hidroxila são capazes de reagir com moléculas celulares e teciduais formando espécies reativas de oxigênio e nitrogênio como peróxido de hidrogênio e óxido nitríco que levam a danos dos elementos celulares vitais, tais como ácidos nucleicos, lípideos e proteínas (BUTTERFIELD et. al , 2010; BUTTERFIELD et al, 2001).

A carbonilação de proteínas é um tipo de oxidação de proteínas que pode ser promovida por espécies reativas de oxigênio (ERO). Geralmente, refere-se a um processo que forma cetonas ou aldeídos reativos que podem reagir com 2,4-dinitrofenil-hidrazina (DNPH) para formar hidrazonas. A oxidação direta de cadeias laterais de lisina, arginina, prolina, e treonina, entre outros aminoácidos são chamadas de reação de carbonilação proteica primária, que leva à formação de um dinitrofenil estável (DNP) produto de uma hidrazona (LEVINE, 2002). A carbonilação de proteínas é um marcador bastante usado para avaliar o estresse sendo o seu aumento observado em diversas patologias incluindo doença de Alzheimer (AD), diabetes e artrite (DALLA-DONE et al., 2003).

O ânion superóxido pode reagir com outros radicais, incluindo espécies de óxido nítrico (NO), produzindo espécies reativas de nitrogênio (ERN) (RADI et al., 2002). Dentre as ERN incluem-se o óxido nítrico (NO•), óxido nitroso ($N_2O_3$), ácido nitroso ($HNO_2$), nitritos ($NO2^-$), nitratos ($NO_3$) e peroxinitritos ($ONOO^-$) (BARREIROS et al., 2006). As ERN podem interagir com componentes mitocondriais, o que leva a uma variedade de respostas biológicas que vão desde a modulação da respiração mitocondrial à morte celular apoptótica. Em

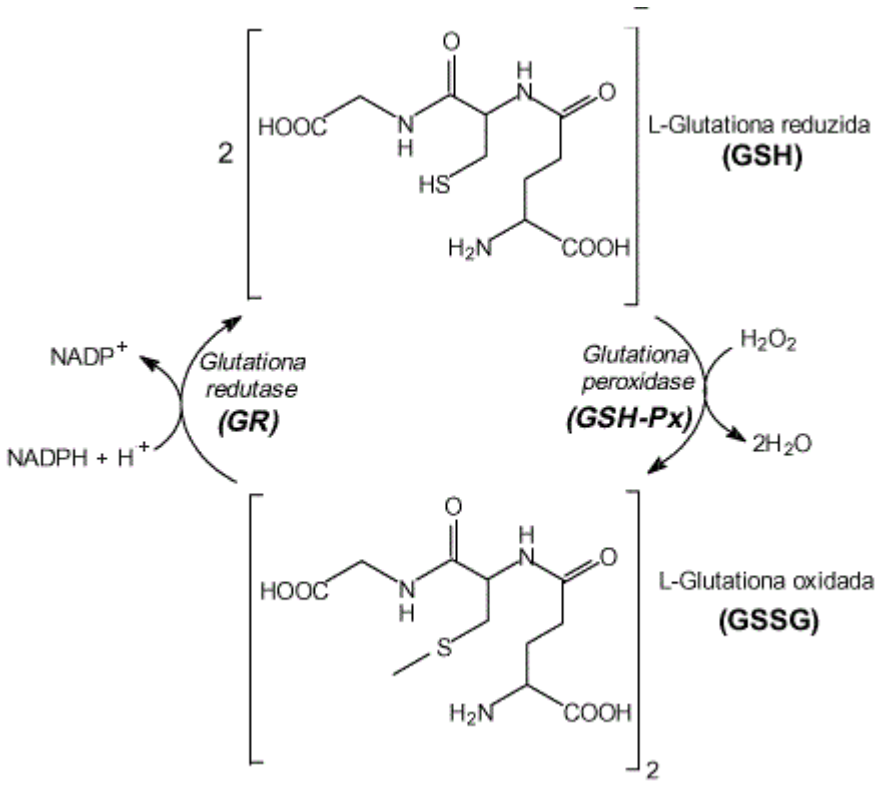
particular, o NO é uma molécula de sinalização que desempenha um papel chave na patogênese da inflamação, como um agente tóxico para os organismos infecciosos ou como imunorregulador (BOGDAN et al, 2000;. BRUNET, 2001). O NO funciona como um mediador pró-inflamatório em baixas concentrações por induzir a vasodilatação e o recrutamento de neutrófilos, enquanto que em elevadas concentrações regula negativamente as moléculas de adesão, suprime a ativação e induz a apoptose de células inflamatórias. Além disso, o NO é um mediador e regulador da função de células *Natural Killer* (NK) que inibe a ativação de mastócitos e pode aumentar ou inibir a ativação de neutrófilos, dependendo da sua concentração (ARMSTRONG, 2001; BIDRI et al, 2001). Esta molécula também induz vasodilatação no sistema cardiovascular e está envolvida em respostas imunes por macrófagos ativados por citocinas (COLEMAN, 2001).

Desta maneira, o estresse oxidativo resulta na acumulação de macromoléculas oxidadas e/ou danificadas que não são removidas e renovadas. Porém, existe um grande sistema de proteção da célula composto de enzimas antioxidantes, tais como a superóxido-dismutase (SOD), a glutationa-peroxidase (GPx) e a catalase (CAT), e outros diversos fatores antioxidantes não-enzimáticos. Uma redução ou uma perda da função das enzimas antioxidantes, pela diminuição de suas atividades específicas, tem sido relatada em doenças neurodegenerativas (KIM et al., 2006; BARATH et al., 2002).

Uma das moléculas essenciais para a defesa antioxidante do organismo é a glutationa reduzida (GSH), que desempenha o importante papel de detoxificação das espécies reativas de oxigênio nas células do SNC. Para o cérebro, o estresse oxidativo tem sido relacionado com a perda de neurônios durante a progressão de doenças neurodegenerativas como, por exemplo, doença de Parkinson (DP), DA, doença de Huntington e acidente vascular cerebral. O metabolismo do GSH no cérebro e suas alterações nas doenças neurodegenerativas já foram previamente estudados (DRINGEN et al., 2000; SCHULZ et al., 2000; BHARAT et al., 2002). Dentro das células, o aumento de ERO altera o equilíbrio redox, afetando a atividade de fatores de transcrição e induzindo vias de sinalização (ADIBHATLA & HATCHER, 2010). Portanto, ocorrem mudanças no ambiente para reduzir a formação de radicais livres, levando a diminuição dos níveis de GSH e os radicais livres produzidos em excesso podem superar as defesas antioxidantes que até então mantiveram a homeostase do ambiente celular (DRIGEN et al., 2000). Durante a desintoxicação de peróxidos a GSH é oxidada formando GSSG, sendo esta reação catalisada pela glutationa peroxidase (GPx). Dentro das células a GSSG é regenerada a GSH a partir da reação catalisada pela glutationa redutase (GR). Durante a ciclagem intracelular redox da glutationa por GPx e GR a GSH é reciclada (Figura

1) (DRIGEN et al., 2003). Assim, a procura por farmácos é relevante para o desenvolvimento de estratégias terapêuticas para diversas doenças em que há um desequilíbrio no estado redox celular.

Figura 2- Interconversão de glutationa nas suas formas reduzida (GSH) e oxidada (GSSH) pela ação das enzimas glutationa peroxidase (GSH-Px) e glutationa redutase (GR)




Fonte: Adaptado de JÚNIOR et al., 2001

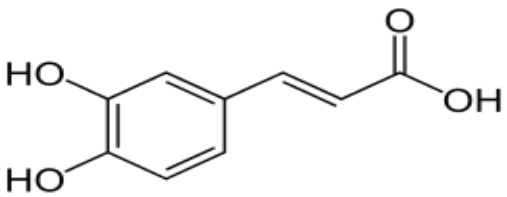
Os compostos fenólicos são conhecidos por oferecerem benefícios terapêuticos devido às suas propriedades antioxidantes e papel modulador na sinalização celular (MENARD et al. 2013, KIM et al. 2014, SCODITTI et al. 2014). Por esta razão, estes compostos são considerados candidatos promissores na prevenção e tratamento de doenças neurodegenerativas, sendo de grande importância investigar as suas propriedades neuroprotetoras. Novas evidências suportam o papel dos polifenóis na prevenção do câncer, nas doenças cardiovasculares, no diabetes e também nas patologias neurodegenerativas (SPAGNINI et al., 2011; SCALBERT et. al., 2005 a,b). Em nosso grupo de pesquisa já foi avaliado o potencial de uma variedade de compostos fenólicos, incluindo o resveratrol,

quercetina, ácido clorogênico, ácido rosmarínico, ácido caféico e antocianinas (SCHMATZ et al., 2009; ABDALLA et al., 2014; SANTI et al., 2014; STEFANELLO et al., 2015; MUSHTAQ et al., 2014; ANWAR et al., 2013; GUTIERRES et al., 2014). Estes compostos tiveram a capacidade de modular a atividade de enzimas, reduzir o estresse oxidativo observados em algumas patologias, bem como reverter danos causados ao SNC.

Portanto, os polifenóis são componentes antioxidantes abundantes na dieta e têm atraído interesse significativo dentro da comunidade científica. Os compostos fenólicos são aqueles que têm, pelo menos, um anel aromático com um ou mais grupos hidroxílicos ligados. Existem milhares de compostos fenólicos ou polifenólicos que são metabolitos secundários de vegetais e, como tal, são encontrados em derivados de plantas, alimentos e bebidas (CROZIER et al., 2009).

Dentre os compostos fenólicos temos o ácido cafeico (AC ácido 3,4-di-hidroxicinâmico) (Figura 2) que é amplamente distribuído em plantas medicinais, frutos, vegetais, vinho, café e óleo de oliva, entre outros, e presente no plasma humano numa concentração dependente da dieta (NARDINI et al.,1998 ;MILES et al., 2005). O AC, livre e esterificado, é geralmente o mais abundante dos ácidos fenólicos e representa 75 a 100% do total dos ácidos hidroxicinâmicos que contém na maioria das frutas, sendo sua concentração aumentada durante o período de maturação diminuindo quanto mais madura a fruta estiver. Já foi descrito um amplo espectro de atividades farmacológicas deste composto incluindo ação anti-inflamatória, antioxidante e efeitos imunomoduladores (ANWAR et al., 2012; GÜLÇIN, 2006; SATO et. al., 2011). O AC pode ser absorvido através do trato gastrointestinal sendo capaz de ultrapassar a BHE e assim exercer suas ações no SNC (OMAR et al., 2011). As propriedades do AC estão presentes uma vez que elimina uma série de espécies reativas, incluindo radicais livres como grupos peroxila e radicais hidroxila (KIKUZAKI et ai, 2002;. GULCIN, 2006, CASTELLUCCIO et ai. , 1995).

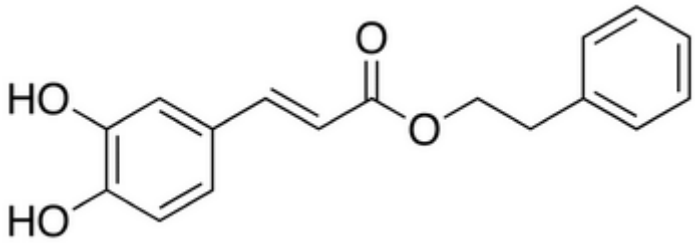
Figura 3 - Estrutura do ácido cafeico




Fonte: Adaptado de KU et al., 2016

O ácido cafeico fenetil éster (ACFE) (Figura 3), assim como o AC, é um composto polifenólico que pode ser sintetizado através da reação entre o AC com fenetil álcoois ou pode ser obtido da própolis, através de extração das colmeias de abelhas (BANKOVA, 2009; KUMAZAWA, 2010; CHEN et al., 2011).

Figura 4 – Estrutura química do ácido cafeico fenetil éster




Fonte: Adaptado de GRUNBERGER et al., 1988

As principais ações do ACFE são devido à presença hidroxilas ligadas ao grupo catecol deste composto que garante uma maior capacidade antioxidante em comparação com outros ácidos fenólicos como, por exemplo, o ácido benzoico (WANG et al., 2006; KURATA et al., 2010). O impedimento estérico das hidroxilas fenólicas por um grupo inerte, tal como um grupo metila, reforça a sua atividade antioxidante através da inibição da propagação das reações de formação de radicais livres (RUSSO et al., 2000, WIDJAJA et al., 2008).

O ACFE é também considerado um potente e específico inibidor da ativação fator nuclear-kB (NF-kB), e isso pode fornecer a base molecular para as suas atividades anti-inflamatórias e imunomoduladoras múltiplas (WANG X et al.,2009; ARMUTCU, F. et al., 2015).

Neste contexto, tendo em vista os inúmeros efeitos benéficos produzidos pelos compostos fenólicos e o envolvimento da neuroinflamação em doenças neurodegenerativas tendo como consequência danos no SNC e formação de ERO, torna-se relevante investigar se o AC e ACFE tem a capacidade de regular as alterações na memória e locomoção e previnir os danos oxidativos em córtex de animais experimentalmente induzidos a inflamação sistêmica por LPS.

## 3 MANUSCRITO CIENTÍFICO

Os resultados que fazem parte desta dissertação apresentam-se sobre a forma de manuscrito científico, que se encontra a seguir estruturado. Os itens materiais e métodos, resultados, discussão e referências, encontram-se inclusos no próprio manuscrito.

**Caffeic acid and caffeic acid phenethyl-ester prevent the redox status impaired by oxidative stress induced by LPS in mice: impacts on cognitive process.**

Diéssica Dalenogare[1], Jessié Martins Gutierres[1], Fabiano Barbosa Carvalho[1,] Pauline Costa[1], Nathieli Bianchini[1], Thauan Faccin Lopes[1], Mariana Sauzen Alves[1], Andressa Bueno[2], Maria Rosa C. Schetinger[1], Vera Maria Morsch[1#]

[1]Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Departamento de Bioquímica e Biologia Molecular, Centro de Ciências Naturais e Exatas; Universidade Federal de Santa Maria, Santa Maria/RS 97105-900, Brasil.

[2]Programa de Pós-Graduação em Medicina Veterinária, Departamento de Pequenos Animais, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria/RS, 97105-900, Brasil.

Corresponding authors.

*Vera Maria Morsch:* Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Departamento de Bioquímica e Biologia Molecular, Centro de Ciências Naturais e Exatas; Universidade Federal de Santa Maria, Santa Maria/RS 97105-900, Brasil.Tel./fax: + 55-55 3220 8978.

*E-mail address:* veramorsch@gmail.com

**Abstract**

This study aims to investigate the effect of caffeic acid (CA) and caffeic acid phenetyl ester CAPE) on redox status and cognitive process in cerebral cortex of lipopolysaccharide-induced mice. Animals were divided into six groups: control; control/AC 50 mg/kg; control/CAPE 30 mg/kg; LPS 250 µg/kg; LPS/CA 50 mg/kg; LPS/CAPE 30 mg/kg. After 30 days of pretreatment with CA or CAPE, the animals received a LPS injection via intraperitoneal. After 24h, the memory of the animals was evaluated by the object recognition task. After the behavioral tasks, the animals were subjected to euthanasia and the cerebral cortex was dissected for the determination of oxidative stress markers (ROS, carbonyl protein and nitrites and nitrates (NOx) levels) and activity of glutathione system and acetylcholinesterase (AChE). Acording to the results only CAPE was able to prevent memory impairment in LPS-induced mice when compared to LPS group. CA and CAPE prevented oxidative damage of protein and also reduced the NOx levels in the cerebral cortex of LPS-induced mice. In addition, both compounds prevented the route of glutathione system triggered by LPS administration. No significant differences were observed between the groups for the activity of the AChE. These findings suggest that CA and CAPE may provide a promising approach for the prevention of redox status caused by systemic inflammation process which impacts on central nervous system.

Key-Words: caffeic acid; caffeic acid phenetyl ester; memory; oxidative stress.

**Introduction**

Peripheral inflammation has been considered to be a trigger of neuropathology onset and progression in several neurodegenerative diseases, since it can cause a brain inflammatory process, also known as neuroinflammation, in which the immune-effector cells of the brain and the infiltrating peripheral immune cells perform a crucial role (Hoogland et al. 2015; Agostinho et al. 2010). During neuroinflammation, the elevated cytokines levels and the oxidative stress trigger neuronal dysfunction, consequently affecting the cognitive function (Dantzer et al. 2008). Increased levels of pro-inflammatory cytokines and oxidative markers were found to be associated with memory impairment associated with dementia neurodegenerative disorders, such as Alzheimer´s disease (Guerreiro et al. 2007).

Lipopolysaccharide is a biologically active membrane component of gram-negative bacteria and is responsible for their toxicity and ability to stimulate the immune system (Lu et al. 2008). The recognition of pathogens is one of the most basic and important property of the immune system that has several specific receptors, such as Toll-like receptors (TLRs). TLRs hyper-stimulation culminates on the increase of nuclear factor kappa B (NF-KB) transcription, upregulation of the pro-inflammatory cytokines expression, such as interleukin IL-1β, IL-6,tumor necrosis factor alpha (TNF-α) and reactive species production (Carvalho et al. 2016; Fruhauf et al. 2015). Furthermore, these deleterious events result in loss of hippocampal neurons and worse learning and memory formation (Carvalho et al. 2016; Fruhauf et al. 2015).

The systemic inflammation by lipopolysaccharide-induced involves diverse molecular mechanisms of inflammation and cellular damage producing oxygen species, such as NO, superoxide anions or peroxynitrite (Kumar et al. 2014; Kawano et al. 2007) and depletion of mitochondrial function (Choumar et al. 2011). Oxygen-derived free radicals are generated during activation of immune system and the antioxidant glutathione (GSH) system is essential for the cellular detoxification of reactive oxygen species in brain. The tripeptide with a sulfhydryl group acts as a cofactor for the antioxidant enzymes, such as glutathione peroxidase (GPx) and glutathione-S-transferase (GST). Furthermore, the GSH system has been connected with the oxidative stress occurring in neurological diseases such as schizophrenia (Do et al. 2000), Alzheimer´s disease (Braidy et al. 2015) epilepsy (Mueller et al. 2001) and Huntington´s disease (Ribeiro et al. 2012).

The oxidative stress production and release of inflammatory mediators in the CNS has been suggested as an important factor for brain pathological events (Carvalho et al. 2016). At the same time, antioxidant agents acting on the reduction of these factors are able to control the deleterious effects in the neuroinflammation. Thus, the discovery of agents that modulate this process can promote an improvement in the prognosis of pathological processes associated with neuroinflammation, such as the progression of neurodegenerative diseases (Allan and Rothwell 2001, 2003).

Caffeic acid (CA) is a common type of phenolic acid, which is frequently found in fruits, coffee, olive oil and Chinese herbal medicines. Most of CA derivatives exist in the form of esters, such

as caffeic acid phenethyl ester (CAPE), a bioactive found in propolis produced by bees (Banskota et al. 2001). CA and CAPE have been reported to present a wide variety of biological activities, including anti-inflammatory, antioxidant and immunomodulatory functions (Deshmukh et al. 2016; Tsai et al. 2015; dos Santos et al. 2014; da Cunha et al. 2004). Recent pharmacological studies have shown that CA exerts a protective effect against hydrogen peroxide-induced oxidative damage in the brain, and prevents behavioral and biochemical alterations in a Alzheimer disease model in rats (Deshmukh et al. 2016). In addition, CAPE also has shown a protective effects against free radicals generation and neurotoxicity induced by 3-nitropropionic acid, a chemical model of Huntington's disease (Deshmukh et al. 2016). CA and CAPE inhibits the activation of NFκB transcription factor, inhibiting the prostaglandins and cyclooxygenases production, which confers on theses bioactive anti-inflammatory and immunomodulatory activities (Kang et al. 2009; Bose et al. 2009; Lee et al. 2004).

Based on neuroprotective evidences of the CA and its derivative CAPE, the present study investigates the ability of these phenolic compounds in preventing the worsening of memory triggered by systemic neuroinflammation. Moreover, it also verifies the activity of regulatory enzymes of the glutathione antioxidant system.

## 2. Materials and Methods

### 2.1 Chemicals

Caffeic acid (CA; >98,0% purity) and caffeic acid phenethyl-ester (CAPE; >97% purity), lipopolysaccharides from *Escherichia coli* (055:B5), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 1-Chloro-2,4-dinitrobenzene 99% (CDNB), L-glutathione oxidized disodium salt 98% (GSSG), β-nicotinamide adenine dinucleotide phosphate reduced tetra (NADPH), cyclohexyl ammonium salt 95%, glutathione reductase from baker's yeast (S. cerevisiae, GR), vanadium (III) chloride, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride (NEED), acetonitrile, 2,4 dinitrophenylhydrazine, 2′,7′-Dichlorofluorescin diacetate were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and of highest purity.

### 2.2 Animals

Male Swiss mice (12 weeks old, 9-10 animals per group) weighing 30–35 g were used in the study. The animals were maintained in the colony cages at an ambient temperature of 23± 2 °C and relative humidity of 45–55% with 12 h light/dark cycles. The animals had free access to a standard rodent pellet diet and water *ad libitum*. All procedures were carried out according to the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care. This work was approved by Ethic Committee on Animal Use of the Federal University of Santa Maria (protocol number 23081.005466/2011-13).

2.3 Experimental protocol and treatments

Mice were pre-treated by gavage with daily oral dose 30mg/kg of CAPE (Bezerra et al. 2012) and 50mg/kg CA (Anwar et al. 2013; Anwar et al. 2012), previously dissolved in corn oil, for 30 days, once a day. On the habituation day to novel object recognition task, the animals received CAPE and CA 2 hours pre-habituation. LPS was dissolved in saline and the selected dose was 250 µg/kg, as described previously (Carvalho et al. 2016). This toxin was administrated by intraperitoneal injection immediately after animal training to impair the memory consolidation. Control group received only vehicle (2 ml/kg of oil, daily by gavage). Mice were randomly distributed into six groups: vehicle, CAPE 30 mg/kg, CA 50 mg/kg, LPS, LPS *plus* CAPE 30 mg/kg and LPS *plus* CA 50 mg/kg. More information can be viewed in the experimental design in figure 1.

2.4 Behavior tasks

2.4.1 Novel Object Recognition Task

The novel object recognition task was performed in a 30 x 30 x 30 cm wooden chamber with three walls painted black and the frontal one made of Plexiglas and the floor covered with ethyl vinyl acetate sheet. A light bulb (60 cm above apparatus) provided constant illumination of about 40 lux and an air-conditioner provided constant background sound isolation. The objects used were pairs of plastic mounting bricks, each pair with different shapes (rectangular, pyramid and stair-like shapes) and colors (white, red and blue), but with the same size. Throughout the experiments objects were used in a random manner, and animals did not display previously preference for any of the objects. Chambers and objects were cleaned after each animal testing with 30% ethanol. The novel object recognition task was performed as previously described (Marisco et al. 2013).

The task consisted in habituation, training and testing sessions, each of them with the duration of 10 min. In the first session, mice were habituated to the apparatus and then returned to their home cage. Twenty-four hours later the training session took place. The animals were exposed to two equal objects (object A), and the exploration time, corresponding to animal´s nose touch or getting close the object (a distance of less than 2 cm) was recorded with two stopwatches. Climbing or sitting on the object was not consider exploration. The test session was carried out 24 h after training. Mice were placed back in the behavioral chamber and one of the familiar objects (i.e. object A) was replaced by a novel object (i.e. object B). The time spent exploring the familiar and the novel objects were recorded. The discrimination index was then calculated, taking into account the time difference spent between exploring the novel (B) and the familiar (A) object x 100 divided by the sum of time spent exploring the novel (B) and the familiar (A), and used as a cognitive parameter ([(Tnovel – Tfamiliar) / (Tnovel X Tfamiliar)] / 100). Vehicle, CAPE and CA were administered 2 hours pre-training of the novel

object recognition task. Saline or LPS (250 µg/kg, intraperitoneally) were administered immediately post-training.

2.4.2 Open-field

The open-field was performed as previously described (Marisco et al. 2013). Immediately after the novel object recognition task, the animals were transferred to a 30 x 30 x 30-cm open field, with the floor divided into 4 squares. During the 10-min open field session, the number of crossing and rearing responses were recorded. The open field was used to identify motor disabilities which might influence the novel object recognition task.

2.5 Samples preparation for biochemical parameters analyzes

The animals were anesthetized and then euthanized. The brain was removed, and the cerebral cortex were separated and further homogenized in a glass potter in a solution of 10 mM Tris–HCl and 0.1 mM EDTA, pH 7.4, on ice. An aliquot of the homogenate was separated. After centrifugation of 1,500 g at 4ºC for 15 min, aliquots of the supernatant were stored at −80 °C until the biochemical analyses.

2.6 Quantification of oxidative stress biomarkers

2.6.1 Carbonyl proteins

Measurement of total protein carbonyl content was determined using the method described by Yan et al. (1995) and adapted for brain tissue by Oliveira et al. (2004). Briefly, cerebral cortex homogenates were adjusted to 0.6 mg/ml of protein in each sample; and 250 µl aliquots were mixed with 50 µl 2,4-dinitrophenylhydrazine (DNPH, 10 mM) or 50 µL ml HCl (2 M). After incubation at room temperature for 1 h (light protected), 125 µl denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing 3 % SDS), 500 µl heptane (99.5 %) and 500 µl ethanol (99.8 %) were added sequentially and further mixed (vortex agitation for 40 s) and centrifuged for 15 min. Afterwards, protein isolated from the interface was washed twice with 500 µL ethyl acetate/ethanol 1:1 (v/v) and suspended in 500 µl of denaturing buffer. Each DNPH sample was read at 370 nm in a Hitachi U-2001 spectrophotometer against the corresponding HCl sample (blank): Total carbonyl levels were calculated using a molar extinction coefficient of 22,000 $M^{-1}$ $cm^{-1}$, as previously described (Levine et al. 1990).

2.6.2. Assay of NOx ($NO_2$ plus $NO_3$)

For NOx determination, the samples were homogenized (1:1) in 200 mM $Zn_2SO_4$ and acetonitrile (Jaques et al. 2013). The homogenates were then centrifuged at 16,000 g for 30 min at 4ºC, and the supernatant was collected for NOx content analysis, as previously described (Miranda et al.

2001). Nitrite and nitrate solutions were used as the reference standards. NOx concentrations were determined by the absorbance at 570 ηm and were expressed, taking into account the standard curve, as µmol of NOx/ mg of protein.

2.6.3 Measurement of ROS levels

The ROS levels were performed using the peroxide production by the cellular components. This analysis is based on the deacetylation of the probe DCFH-DA, and its subsequent oxidation by reactive species to DCFH, a highly fluorescent compound. The homogenate was added to a medium containing Tris–HCl buffer (10 mM; pH 7.4) and DCFH-DA (1 mM). After DCFH-DA addition, the medium was incubated in the dark for 1 h until the start of fluorescence measurement procedure (excitation at 488 nm and emission at 525 nm, and both slit widths used were at 1.5 nm). DCF levels were determined using a standard curve of DCF, and results were corrected by the protein content (Myhre et al. 2003). The results were expressed by U DCFH/mg of protein.

2.7 Glutathione enzymatic system

2.7.1 Glutathione reductase (GR)

GR activity was determined as described by Carlberg and colleagues (Carlberg and Mannervik 1985). The method is based on using the oxidized enzyme glutathione (GSSG), to convert GSSG in GSH in the presence of the cofactor NADPH. Briefly, cerebral cortex supernatant (15µl) was added to medium containing 0.2 M phosphate buffer (0.2 M $K_2HPO_4$ and 2 mM EDTA, pH 7.0) and NADPH (2 mM). The reaction was initiated by adding substrate GSSG (20 mM). The measurement of GR levels was accomplished by absorbance at 340 nm during 2 min of incubation. GR activity was determined using the molar extinction coefficient $6220\ M^{-1}\ cm^{-1}$ and expressed as ηmol NADPH/min/mg of protein.

2.7.2 Glutathione peroxidase (GPx) activity

GPx activity was determined using cerebral cortex supernatant, glutathione reductase and NADPH. The method is based on the oxidation of NADPH, which is indicated by a decrease in absorbance at 340 nm (Paglia and Valentine 1967). The enzymatic activity was expressed as ηmol $H_2O_2$/min/mg of protein.

2.7.3 Glutathione S-Transferase (GST) activity

GST activity was assayed spectrophotometrically at 340 nm by the method developed by Habig and colleagues (Habig et al. 1974). The mixture contained cerebral cortex supernatant as test, 0.1 M potassium phosphate buffer (pH 7.4), 100 mM GSH and 100 mM CDNB, which was used as substrate. The enzymatic activity was expressed as ηmol CDNB/min/mg of protein.

2.9 Protein determination

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

2.9 Statistical analysis

Statistical analyses of tests were carried out by one-way ANOVA, followed by post hoc Tukey Test. $P<0.05$ was considered to represent a significant difference in all experiments. All data were expressed as the mean ± SEM.

## 3. Results

3.1. CAPE prevents impairment of memory induced by systemic LPS administration.

Figure 2 shows the effect of CA and CAPE on long-term memory of mice 24 hours after systemic administration of LPS. Graph 2A shows that the pre-treatment with CA was not able to prevent the memory deficits induced by LPS. However, CAPE was able to protect against the memory loss induced by LPS [$F_{(5,50)}=4.929$, $P<0.001$; graph 2A]. In relation to locomotor (graph B) and exploratory (graph C) parameters, it was not observed changes in the crossing [$F_{(5,50)}=0.266$, $P>0.05$; graph 2B] and rearing [$F_{(5,50)}=0.206$, $P>0.05$; graph 2C] numbers.

3.2. CA and CAPE prevented the oxidative stress induced by LPS in cerebral cortex.

Upon 24 hours of intraperitoneal LPS-administration, the protein carbonyl and NOx content increased in cerebral cortex [$F_{(5,50)}= 4,784$, $P<0.001$; graph 3A and $F_{(5,50)}= 17.20$, $P<0.001$; graph 3B, respectively]. The same effect was observed in ROS levels compared to vehicle group [$F_{(5,50)}= 3.772$, $P <0.05$; graph 3C]. CA and CAPE were able to prevent an increase in carbonyl protein and NOx levels. Only CAPE showed effectiveness in protect against the increase in the ROS levels induced by LPS.

3.3 CA and CAPE prevented changes in the glutathione system triggered by LPS administration in the cerebral cortex.

The intraperitoneal LPS-administration decreased the GR activity in the cerebral cortex. However, CA and CAPE were able to restore the GR activity [$F_{(5,50)}=3.647$, $P<0.05$, graph 4A]. It was observed an increase in the GPx activity when LPS and vehicle group were compared. The CA and CAPE prevented the increase in the GPx activity when compared to LPS group [$F_{(5,50)}=3.647$, $P<0.05$, graph 4B]. Moreover, LPS group increased the GST activity and CA and CAPE were able to protect this effect [$F_{(5,50)}=3.432$, $P<0.05$, graph 4C].

## 4. Discussion

The present study investigated the neuroprotective properties of the CA and its derivative, CAPE, in protecting memory deficits and oxidative stress triggered by lipopolysaccharide injection. In parallel, it was also verified the activity of regulatory enzymes of glutathione system. We found that pretreatment during 30 days with CAPE was able to prevent memory impairment (assessed though the object recognition task) triggered by intraperitoneal lipopolysaccharide injection.

Studies from our group showed that CA intake can improve the performance of rats in the inhibitory avoidance task (Anwar et al. 2012). In fact, after this evidence, several researches emerged, showing a potential effect of CA as a nootropic agent. Briefly, it was observed that CA protects cognitive deficits induced by focal (Pinheiro Fernandes et al. 2014) and global cerebral ischemia (Liang et al. 2015). Next, promising evidence were also seen in two experimental models to Alzheimer disease, using β-amyloid peptide (Kim et al. 2015) and streptozotocin (Deshmukh et al. 2016). In this study, we did not observe a protective effect of CA against memory deficits induced by LPS. Furthermore, CA also did not show a *per se* effect on learning and memory formation. This effect can be related to the type of task used in this study, as well as the memory associated with the object recognition. Memories associated with aversive stimuli, such as that formed in the inhibitory avoidance task, are less labile and easier to consolidate when compared to the type of memories formed in the object recognition (Cammarota et al. 2007; Alonso et al. 2002; Clarke et al. 2010). Interestingly, CAPE showed the ability to protect the impairment of memory resulting from LPS administration in mice .

The oxidative stress induced by LPS promotes the release of cytokines, prostanoids and reactive species. The sum of these deleterious events culminates in loss of hippocampal neurons, worse learning and memory (Carvalho et al. 2016; Valero et al. 2014). Reports also indicate that a chronic state induced by LPS contributes to formation of beta-amyloid peptide (Lee et al. 2008) and the development of Alzheimer's disease (Miklossy 2008; Jaeger et al. 2009). As a result, the discovery of agents that modulate neuroinflammation may promote an improvement in the prognosis of pathological processes associated with this disease.

In the brain, the high content of polyunsaturated fatty acids and the high oxygen consumption are factors responsible for elevated susceptibility to reactive species damage, with impact on the development of several neurodegenerative (Vida et al. 2014; Sutherland et al. 2013; Sultana et al. 2013). The systemic administration of LPS promoted an increase in the markers of oxidative/ nitrosative damage, such as the formation of carbonyl protein, NOx and ROS total levels in cerebral cortex. These data are in accordance with other studies that showed an increase in the oxidative stress markers after LPS administration (Vasconcelos et al. 2015; Swarnkar et al. 2009; Abdel-Salam et al. 2014). Our data also showed that the oral administration of CA and CAPE was able to prevent the oxidative damage of proteins and also reduce the NOx levels induced by LPS, suggesting that these compounds have antioxidant properties against damage caused by LPS administration.

Enzymes of glutathione system are able to neutralize reactive species and can protect cells from damage induced by hydrogen peroxide. In the present study, the LPS caused a decrease in GR and an increase in GPx and GST activities. Next, it was observed that both CA and CAPE restore the route of glutathione system. Since CA and CAPE are scavenger and neutralize reactive species, these compounds can restore GSH levels and improve the activity of glutathione system. Albukhari and collegues found that CAPE intake is able to increase GSH biosynthesis in liver of rats (Albukhari et al. 2009). In parallel, other hypothesis to explain the protective effect is that CA and CAPE can reduce the production of reactive species by suppressing the secretion of pro-inflammatory mediators. In line with this, CAPE also suppresses the inflammatory response in vitro (Choi et al. 2015) reducing the NOx increased, iNOS expression and the IL-1β and IL-6 levels in macrophages and microglial cells stimulated by LPS. In addition, CAPE also decreased nuclear translocation of NF-KB p65 and p50 subunits induced with LPS in macrophages (Choi et al. 2015; Tsai et al. 2015). Based on this evidence, it is possible to assume that these compounds can reduce the oxidative stress induced by LPS in the central nervous system.

Our findings indicate that CA and CAPE were able to protect against the production of oxidative and nitrosative stress in the cerebral cortex of mice and protect the activity of enzymes that comprise the route of glutathione system. However, only CAPE was able to protect memory in mice exposed to LPS. These findings show a potential beneficial effect against oxidative stress induced by lipopolysaccharide however more studies should be done to better understand the mechanisms of action of CA and CAPE as bioactive compounds.

**Conflicts of Interest statement**

There are no conflicts of interest.

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**Listo of Legends**

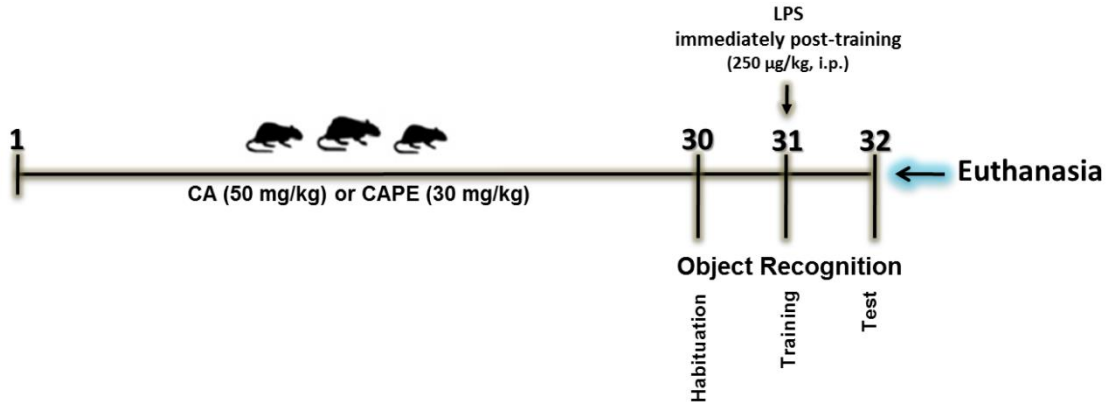


**Figure 1.** *Experimental design:* Mice were pre-treated by gavage daily with an oral dose of CAPE (30mg/kg) or CA (50mg/kg) during 30 days (once a day). On the 30th occurred habituation to object recognition task. The animals received CAPE and CA 2 hours pre-habituation. LPS (250 µg/kg) was dissolved in saline and administered by intraperitoneal injection immediately after animal training

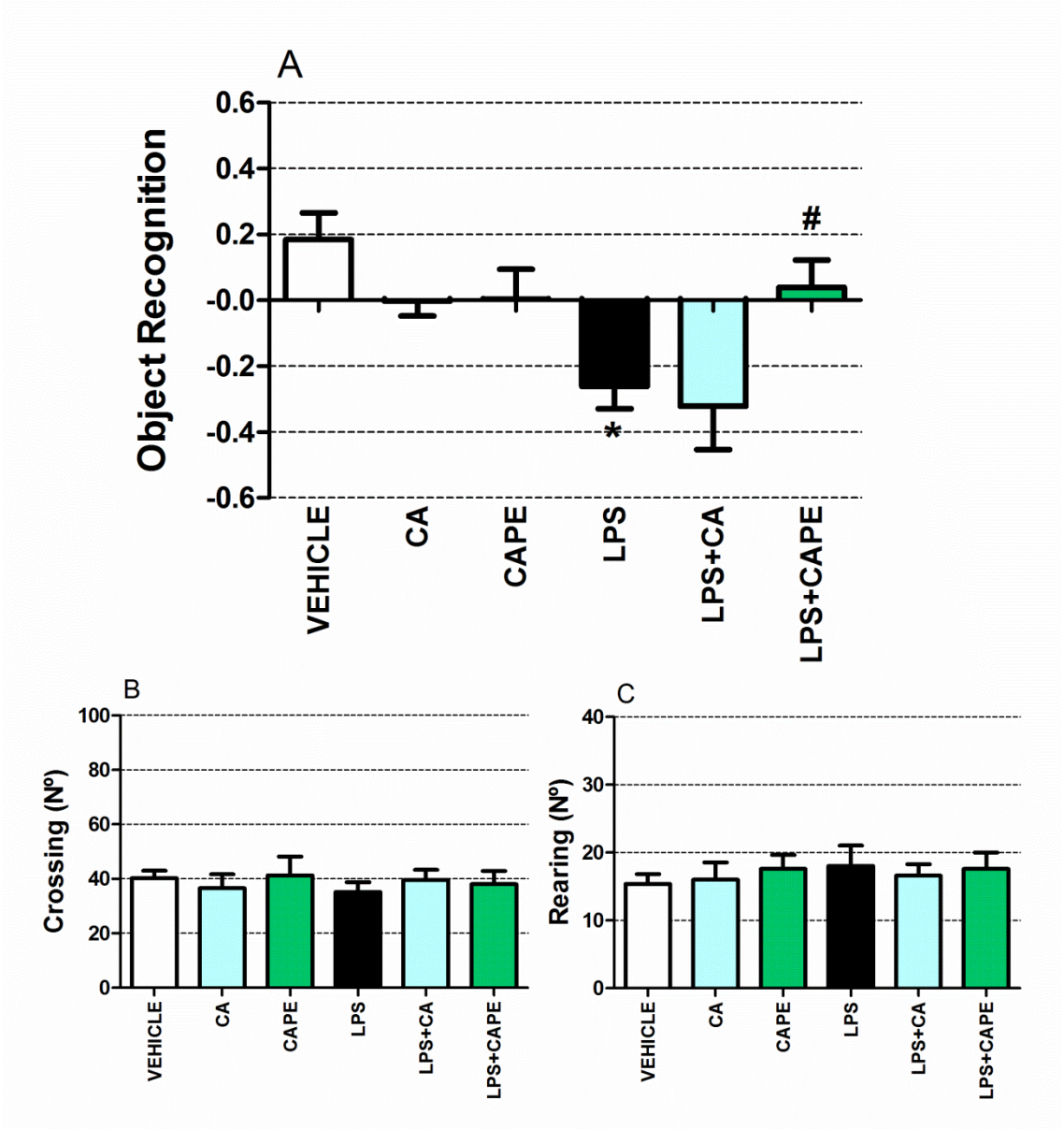


**Figure 2.** Effects of caffeic acid (CA, 50 mg/kg) and caffeic acid phenethyl-ester (CAPE, 30 mg/kg) intake on the memory loss induced by LPS (250 µg/kg) injected intraperitoneally (i.p.) in mice, assessed by the object recognition task (graph A). Effects of the treatments on the crossing (C) and rearing numbers (D). Data are expressed as mean ± SEM of 9-10 independent animals. $P<0.05$ was considered to represent a significant difference. *Denotes significant different from the vehicle group. # Denotes a significant difference compared with the LPS group (ANOVA one-way followed by post-hoc Tukey Test. (*,#Denotes significant difference $P<0.05$).

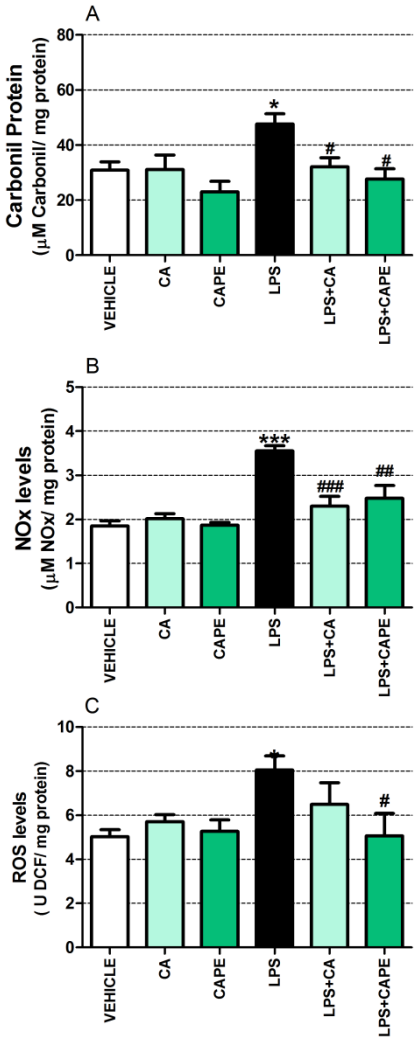


**Figure 3.** Effects of caffeic acid (CA, 50 mg/kg) and caffeic acid phenethyl-ester (CAPE, 30 mg/kg) intake on the oxidative stress markers in the cerebral cortex of mice treated with LPS (250 µg/kg) injected intraperitoneally (i.p.). (A) Carbonyl protein content, (B) NOx levels and (C) ROS levels. Data are expressed as mean ± SEM of 9-10 independent animals. $P<0.05$ was considered to represent a significant difference. *Denotes significant different from the vehicle group. # Denotes a significant difference compared with the LPS group (ANOVA one-way followed by post-hoc Tukey Test. (*,# Denotes significant difference $P<0.05$, **,## Denotes significant difference $P<0.01$, ***,### Denotes significant difference $P<0.001$).

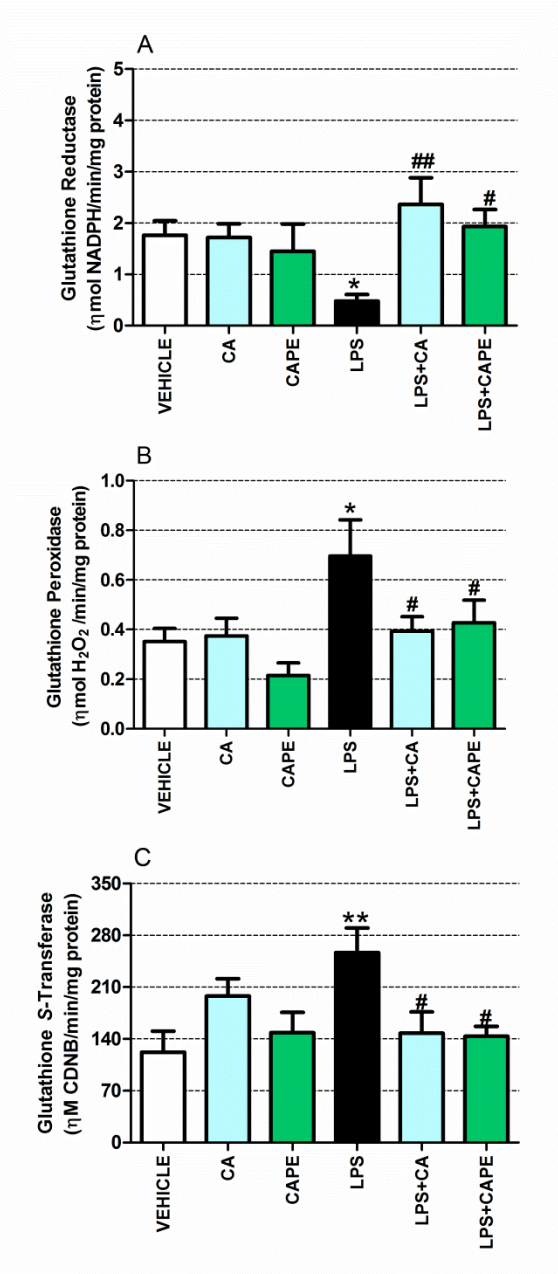


**Figure 4.** Effects of caffeic acid (CA, 50 mg/kg) and caffeic acid phenethyl-ester (CAPE, 30 mg/kg) intake on the activity of glutathione system enzymes in the cerebral cortex of mice treated with LPS (250 µg/kg) injected intraperitoneally (i.p.). (A) Glutathione reductase (GR), (B) glutathione peroxidase (GPx) and (C) glutathione-S-transferase (GST) activities. Data are expressed as mean ± SEM of 9-10 independent animals. $P<0.05$ was considered to represent a significant difference. *Denotes significant different from the vehicle group. # Denotes a significant difference compared with the LPS group (ANOVA one-way followed by post-hoc Tukey Test. (*,#Denotes significant difference $P<0.05$, **,##Denotes significant difference $P<0.01$).

## 4 CONCLUSÃO

Através dos resultados obtidos no presente estudo pode-se concluir que o ácido cafeico fenetil éster não altera a atividade locomotora dos camundongos, porém é capaz de ter um efeito benéfico sobre a memória que é prejudicada pelo modelo de inflamação em camundongos induzido por LPS. Desta forma, sugere-se que o ACFE através de suas propriedades antioxidantes e anti-inflamatórias pode reduzir os danos causados pelo modelo de inflamação que resulta em um processo neuroinflamatório tendo efeito benéfico à memória dos camundongos que receberam pré-tratamento com este composto fenólico.

Além disso, também se pode observar o efeito antioxidante de ambos compostos, AC e ACFE, sobre os parâmetros de estresse oxidativo tendo uma diminuição da carbonilação proteica, os níveis de nitrito e nitrato e também de espécies reativas de oxigênio. Os compostos fenólicos apresentaram ainda efeito restaurador no sistema antioxidante da glutationa.

O AC e ACFE mostram efeitos benéficos contra o estresse oxidativo sendo, portanto compostos promissores nos estudos relacionados a doenças neurodegenerativas que envolvam o processo onde há um desequilibro no estado redox no SNC, necessitando de uma investigação mais detalhada de seus mecanismos de ação.

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**ANEXO A -** Carta de aprovação pelo Comitê de Ética - UFSM

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

# CERTIFICADO

Certificamos que o Projeto intitulado "Participação do receptor P2X7 na inflamação induzida por lipopolissacarídeo: Efeitos do Ácido Cafeico Fenetil Ester e Ácido Cafeico", protocolado sob o CEUA nº 8348100315, sob a responsabilidade de **Vera Maria Melchiors Morsch** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei 11.794, de 8 de outubro de 2008, com o Decreto 6.899, de 15 de julho de 2009, com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais Universidade Federal de Santa Maria (CEUA/UFSM) em reunião de 21/05/2015.

We certify that the proposal "Participation of P2X7 receptor in lipopolyssacharide induced inflammation: caffeic acid phenethyl ester and caffeic acid", utilizing 77 Heterogenics mice (77 males), protocol number CEUA 8348100315, under the responsibility of **Vera Maria Melchiors Morsch** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes (or teaching) - it's in accordance with Law 11.794, of October 8 2008, Decree 6899, of July 15, 2009, 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 05/21/2015.

Vigência da Proposta: de 03/2015 a 12/2016 Laboratório: Bioquímica/departamento De Bioquímica E Biologia Molecular

| | | | | | |
|---|---|---|---|---|---|
| Procedência: | Biotério Central UFSM | | | | |
| Espécie: | Camundongos heterogênicos | Gênero: Machos | idade: | 60 DIAS | N: 77 |
| Linhagem: | C57BL6 | | Peso: | 20-30g | |

Nota: Os nucleotídeos e nucleosídeo de adenina são considerados importantes mediadores inflamatórios, sendo o ATP um regulador de uma variedade de processos celulares, através da ativação de receptores do tipo P2Rs. O receptor P2X7 merece destaque por exercer suas funções principalmente quando há alguma lesão ou processo inflamatório, podendo estar relacionado a várias doenças infecciosas, inflamatórias e cardiovasculares. Dada à sua importância nas funções do organismo, é de grande relevância a caracterização da presença e da função deste receptor na geração de antagonistas seletivos e não-seletivos com potencial terapêutico. Estudos tem evidenciado que alguns polifenóis podem representar um importante papel no controle do processo inflamatório no câncer, nas doenças cardiovasculares, diabetes e patologias neurodegenerativas. Dentre esses polifenóis estão o ácido caféico (AC) e o ácido cafeico fenetil éster (ACFE). O objetivo deste trabalho será avaliar o potencial terapêutico destes dois compostos na regulação do receptor P2X7 avaliando parâmetros inflamatórios, bioquímicos e moleculares no modelo experimental de inflamação sistêmica induzido por lipopolissacarídeo. Um total de 77 camundongos serão divididos em sete grupos(n=11): controle/salina, controle/AC-10 mg/kg, controle/ACFE-15 mg/kg, LPS 250µg/kg, LPS/AC-10 mg/kg, LPS/ACFE-15 mg/kg e LPS/BBG-50 mg/kg tratados via oral, diariamente, durante 15 dias, sendo a injeção de LPS administrada após 15 dias de tratamento.

Santa Maria, 25 de maio de 2015

Vania Lucia Loro

Profa. Dra. Vânia Lucia Loro
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Avenida Roraima, 1000, Reitoria, 2º andar - CEP 97105-900 Santa Maria, RS - tel: 55 (55) 3220-9362 / fax: 55 (55) 3220-8009
Horário de atendimento: das 8:30 ás 11:30 e 14:00 ás 16:30hs : e-mail: ceua.ufsm@gmail.com
CEUA Nº 8348100315