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PARÂMETROS COMPORTAMENTAIS E NEUROTÓXICOS DE GAMA-DECANOLACTONA EM MODELOS EXPERIMENTAIS DE EPILEPSIA, ESTRESSE OXIDATIVO E GENOTOXICIDADE

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RESUMO

Gama-decanolactona (GD) é um composto monoterpeno com estrutura semelhante às lactonas naturais, que estão presentes em diferentes espécies e são usadas como terapia farmacológica na região amazônica. GD mostrou um efeito anticonvulsivo em ambos os modelos de pentilenotetrazol (PTZ) agudo e crônico e atua como um antagonista de glutamato não-competitivo. Considerando estudos anteriores sobre atividades biológicas de GD, o presente estudo teve como objetivo explorar o seu perfil anticonvulsivante em diferentes modelos de epilepsia em camundongos e sobre parâmetros de estresse oxidativo, neuroinflamação e genotoxicidade em células microgliais N9. A atividade anticonvulsiva de GD (100 e 300 mg/kg) foi investigada em convulsões induzidas por aminofilina, isoniazida, picrotoxina, 4-aminopiridina (4-AP) e pilocarpina em camundongos machos. Os animais receberam uma administração de solução salina (SAL), GD ou diazepam (DZP 2 mg/kg), de controle positivo e após 30 min receberam os seguintes agentes convulsivos: aminofilina, isoniazida, picrotoxina, 4-AP ou pilocarpina. Os parâmetros avaliados durante 1h foram a latência para a primeira convulsão, a porcentagem de convulsões e a taxa de mortalidade. A fim de avaliar a neurotoxicidade de GD 100 e 300 mg/kg, foi realizado o teste de desempenho de rotarod. O tempo para cair do rotarod foi gravado em diferentes momentos após a administração GD. E por último foi avaliado o desempenho de GD 100 e 300 mg/kg no teste de sono induzido por DZP (17 mg/kg). Os resultados demonstraram que GD foi capaz de aumentar a latência para a primeira convulsão induzida pela aminofilina, isoniazida, 4-AP e pilocarpina, mas não para picrotoxina. No teste de rotarod, GD 300 mg/kg reduziu o tempo de latência para cair da barra após 30 min de administração, mas não após 60, 90 ou 120 min. GD 300 mg/kg aumentou a latência do sono induzido pro DZP. Os resultados acima revelaram que GD apresentou um efeito anticonvulsivo nos modelos de epilepsia utilizados neste estudo, principalmente na dose de 300 mg/kg, bem como o tempo de latência para a primeira convulsão, sugerindo que GD pode modular as vias da adenosina e afetar os canais de potássio, direta ou indiretamente. O papel de GD sobre o estresse oxidativo em *status epilepticus* (SE) foi investigado medindo a produção de espécies reativas de oxigênio (EROS), superóxido dismutase (SOD), catalase (CAT), teor de nitrito, e os danos do DNA induzidos por pilocarpina no córtex cerebral de camundongos. GD foi capaz de

aumentar as atividades de SOD e CAT, diminuir a produção de EROS, NO e danos no DNA durante o estabelecimento de SE no córtex cerebral. Estes dados sugerem que as enzimas SOD e CAT são o principal sistema de eliminação de radicais livres, e GD fornece neuroproteção contra o aumento do estresse oxidativo no cérebro.

Um modelo clássico para investigar se uma droga atua modulando a neuroinflamação é utilizar a linhagem celular N9 (célula neuronal da microglia murina) ativada com o químico lipopolissacárideo (LPS), que sabidamente, provoca inflamação. Assim, investigou-se o efeito da GD sobre parâmetros inflamatórios e apoptóticos nesse sistema. Avaliou-se a expressão protéica por western blotting das seguintes proteínas: óxido nítrico sintetase induzível (iNOS) e fator de necrose tumoral-alpha (TNF-α), p-38 fosforilada e não fosforilada. Avaliou-se também a atividade da caspase 9-clivada e a formação de EROS por citometria de fluxo bem como o dano ao DNA pelo ensaio cometa alcalino. Nos resultados *in vitro,* GD atenuou a ativação de células N9 microglias, inibiu a formação de EROS intracelular e a expressão de iNOS e TNF-α induzidas por LPS nas células. Além disso, GD bloqueou a fosforilação da p38, inibiu a caspase-9 clivada e o dano ao DNA. Estes dados indicam que GD tem um potencial terapêutico neuroprotetor e que exerce os seus efeitos através da inibição da inflamação.

ABSTRACT

Gamma-decanolactone (GD) is a monoterpene compound similar to natural lactones in structure, which are present in different species and are used as remedy in the Amazonian region. GD shows an anticonvulsant effect in both the acute and chronic pentileneterazole (PTZ) models and acts as a non-competitive glutamate antagonist. Considering previous studies on biological activities of GD, the present study aimed to explore its anticonvulsant profile in different epilepsy models in mice and on oxidative stress parameters in N9 cell. The anticonvulsant activity of GD (100 and 300 mg/kg) was investigated on seizures induced by aminophylline (AMPH), isoniazid (INH), picrotoxin (PCT), 4-aminopyridine (4-AP) an pilocarpine (PIL) in male mice. The animals received one administration of saline (SAL), GD or the positive control diazepam (DZP 2 mg/kg), and after 30 min they received the following convulsant agents: AMPH, INH, PCT, 4-AP or PIL The parameters evaluated during 1h were the latency to first seizure, the occurrence of seizure and the mortality rate. In order to evaluate the neurotoxicity of GD 100 and 300 mg/kg, the rotarod performance test was performed. The time to fall of the rotarod was recorded at different times after the GD administration. The results demonstrated that GD was able to extend the latency to first seizure induced by AMPH, INH, 4-AP and PIL, but not PCT. In the rotarod test GD 300mg/kg reduced the latency to fall of the bar just at 30 min after the administration, but not at 60, 90 or 120 min. The above results revealed that GD presented an anticonvulsant effect in the epilepsy models used in this study, especially at the dosage of 300mg/kg, as well as the latency to the first seizure, suggesting that the GD could modulate the adenosine and GABA pathways and affect potassium channels directly or indirectly. The role of gamma-decanolactone (GD) on oxidative stress in pilocarpine-induced SE was investigated by measuring ROS production, superoxide dismutase and catalase activities, nitrite content, and DNA damage in the mice cerebral cortex. GD was able to increase the superoxide dismutase (SOD) and catalase (CAT) activities, decreased the ROS, NO production, and DNA damage during the SE establishment in the cerebral cortex. These data suggest that the cortex use SOD and CAT enzymes as the major free-radicals scavenging system, and GD provides neuroprotection against the increase of oxidative stress in the brain. Because previous works have consistently demonstrated the N9 microglial-neuronal system for neuroinflammation

investigations, these cells are considered appropriate for this kind of research. To investigate the inhibitory effect of GD on the production of ROS and inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS) - stimulated N9 murine microglial cells through the p38 MAPK signaling pathway. The results in vitro the GD attenuated the activation of N9 cells and inhibited intracellular ROS and the expression of iNOS and TNF-α induced by LPS in the cells. In addition, GD blocked the phosphorylation of p38 and inhibited cleaved caspase-9 and DNA damage. These data indicate that GD has therapeutic potential for the treatment of neurodegenerative diseases, and that it exerts its effects by inhibiting inflammation.

LISTA DE ABREVIAÇÕES E FÓRMULAS

AMPA - alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiônico

ATP – Adenosina Trifosfato

CAT – Catalase

DZP – Diazepam

ELT - Epilepsia do Lobo Temporal

EROS – Espécies Reativas de Oxigênio

ERNS – Espécies Reativas de Nitrogênio

GABA – Ácido Gama-aminobutírico

GAD – Glutamato Descarboxilase

GD – Gama-decanolactona

GPx – Glutationa Peroxidase

GR – Glutationa Redutase

GST - Glutationa-S-Transferase

H_2O_2 – Peróxido de Hidrogênio

IL-1β - Interleucina-1β

IL-10 - Interleucina-10

IL-6 - Interleucina-6

Iba-1- Ionized calcium binding adaptor molecule

LBE - Liga Brasileira de Epilepsia

LPS – Lipopolissacarídeo

NO – Óxido Nítrico

NMDA - N-metil-D-aspartato

iNOS - Óxido Nítrico Sintase Induzível

O_2^- - Radical Ânion Superóxido

$^{\cdot}OH$ – Radical hidroxil

$ONOO^{-}$ - Peroxinitrito

PTZ- Pentilenotetrazol

SE – Estado Epiléptico

SNC – Sistema Nervoso Central

SOD – Superóxido Dismutase

TNF-α – Fator de Necrose Tumoral-alpha

4-AP – 4-Aminopiridina

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1 INTRODUÇÃO

1.1EPILEPSIA

Epilepsia é uma desordem neurológica crônica, caracterizada por descargas neuronais excessivas e anormais no cérebro, que levam a episódios convulsivos espontâneos e recorrentes (SHIHA *et al*., 2014; WANG *et al*., 2016). As crises epilépticas podem estar associadas a uma variedade de sintomas clínicos como alterações da consciência, movimentos e sensações (CHINDO *et al*., 2014). O mecanismo neuronal envolvido na epilepsia ainda não está totalmente elucidado, mas sabe-se que há um desequilíbrio entre os principais sistemas de neurotransmisão excitatório (glutamatérgico) e inibitório (gabaérgico) (EYO *et al*., 2016). Epilepsia é uma das desordens cerebrais existentes mais comuns e a sua prevalência na população em geral é de aproximadamente 1 a 2% (SHIHA *et al*., 2014; S´WIADER *et al*., 2016) atingindo, pelo menos, 70 milhões de pessoas em todo o mundo (SUCHER E CARLE, 2015) e cerca de 3 milhões de brasileiros, segundo a Liga Brasileira de Epilepsia (LBE, 2016) e Organização Mundial da Saúde (OMS, 2016).

Muitos fármacos antiepilépticos estão disponíveis na clínica, porém, cerca de 30% dos pacientes são refratários aos tratamentos disponibilizados até o momento, levando em conta que os medicamentos existentes são mais específicos para suprimir ataques convulsivos e não para tratar mecanismos fisiopatológicos em si. Outro agravante relacionado ao uso de medicamentos antiepilépticos são os efeitos adversos provocados durante o uso, como: sedação, depressão, comportamento aberrante, irritabilidade, impulsividade, raiva, hostilidade e agressão (BRODIE *et al*., 2016). Com isso a busca de um melhor entendimento do processo da epileptogênese, se torna profícua e pode levar ao desenvolvimento de novos fármacos mais eficazes e com menos efeitos adversos (VRIES *et al*., 2016).

1.1.1 Classificações da Epilepsia

De acordo com a etiologia, a epilepsia pode ser dividida em três categorias principais: Idiopática, Adquirida e Criptogênica. Epilepsia Idiopática é uma crise sem lesão cerebral subjacente ou ainda sem sinais e sintomas neurológicos, que se

presume ser genética ou com inicio na infância. Epilepsia Adquirida é denominada como sendo crises epilépticas, provenientes de uma ou mais lesões estruturais do cérebro. Epilepsia Criptogênica, refere-se à epilepsia sintomática, sem causa conhecida. Entre os casos descritos de epilepsia, aproximadamente 40% possuem etiologia conhecida, incluindo lesão cerebral traumática, acidente vascular cerebral isquêmico, hemorragia intracerebral, infecções do sistema nervoso central, tumores cerebrais, várias doenças neurodegenerativas e prolongadas convulsões agudas sintomáticas tais como convulsões febris complexas ou SE (LÖSCHER E BRANDT, 2010; YOW *et al*., 2013).

A classificação clínica das crises epilépticas é feita com base nas características das crises convulsivas. Existem duas categorias principais divididas em: Crises parciais e crises generalizadas que ainda podem ser divididas em simples (sem perda da consciência) ou complexas (com alteração da consciência). Crises parciais são aquelas em que a despolarização ocorre localmente em um dos hemisférios e as lesões cerebrais são focais. A epilepsia parcial mais frequentemente encontrada é a epilepsia do lobo temporal (ELT) que atinge pelo menos 20% dos pacientes epilépticos e é considerada a mais refratária dentre as existentes (REDDY E KURUBA, 2013).

Crises generalizadas envolvem o cérebro inteiro, produzindo lesões em ambos os hemisférios e uma das características seria a perda de consciência imediata. Existem muitos tipos de crises generalizadas, sendo que as mais importantes são as tônico-clônicas (antigamente denominadas de ‘’grande mal’’), onde ocorre contração tônica e sustentada de toda a musculatura corporal e as crises de ausência (‘’pequeno mal’’) onde a pessoa se encontra ‘’desligada” por alguns instantes, podendo retomar o que estava fazendo em seguida. Existem ainda convulsões atônicas, manifestadas como perda súbita do tônus muscular postural com duração de 1 a 2 segundos, as clônicas que se caracterizam por várias contrações musculares do corpo ou membros com duração de poucos segundos ou minutos e por último as mioclônicas que se caracterizam por breves abalos musculares deixando cair objetos das mãos (RANG *et al*., 2012; VRIES *et al*., 2016).

1.1.2 Epileptogênese

O termo epileptogênese é usado para descrever um processo que leva à ocorrência da primeira crise espontânea e eventos epileptiformes recorrentes após o insulto cerebral, passando de um cérebro normal para anormal (YOW *et al*., 2013). Segundo estudo realizado por Reddy e Kuruba (2013) a epileptogênese pode ser dividida em três estágios (Fig. 1): (1) evento inicial, (2) período de latência, (3) período crônico com crises espontâneas. Epileptogênese é um processo lento e leva vários meses para crises espontâneas aparecer, sendo o período de latência o momento para a ocorrência de convulsões. Para desempenhar um papel central na patogênese da epilepsia geral, tem sido propostos modelos de neuroinflamação e lesões neuronais conforme descrito na figura 1.

Figura 1- A base fisiopatológica da epileptogênese, seguido de episódio de SE.

Fonte: Adaptado de Reddy and Kuruba, 2013.

Evidências sugerem que um dos fatores que podem contribuir para o processo da epileptogênese é a neuroinflamação, envolvendo a ativação das células gliais e um aumento de mediadores inflamatórios específicos (WANG *et al*., 2015). Outro dado importante é que o estresse oxidativo-nitrosativo também é considerado como possível mecanismo envolvido na patogênese da epilepsia (AGUIAR *et al*., 2012).

1.1.3 Fisiopatologia da Epilepsia

Apesar da fisiopatologia da epilepsia ainda ser pouco compreendida, como já mencionado anteriormente, sabe-se que há um desequilibro no mecanismo excitatório e inibitório do sistema nervoso central (SNC). Ambos os neurotransmissores glutamato e ácido gama-aminobutírico (GABA), possuem um papel crucial no fenômeno da epilepsia (EYO *et al*, 2016).

Glutamato é o principal neurotransmissor responsável pelo potencial pós-sináptico excitatório, por despolarização dos neurônios. Receptores glutamatérgicos são divididos em ionotrópicos, ligados a canais de Ca^{2+} (N-metil-D-aspartato (NMDA), alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiônico (AMPA) e Cainato) e metabotrópicos (acoplados a proteína G). O mecanismo molecular glutamatérgico envolvido na epilepsia consiste na regulação positiva dos receptores de glutamato, elevação da concentração extracelular de glutamato e anormalidade no transporte glutamatérgico. Esses fenômenos contribuem para o papel da hiperexcitabilidade na epilepsia (YOW *et al*., 2013; BARKER-HALISKI *et al*, 2015).

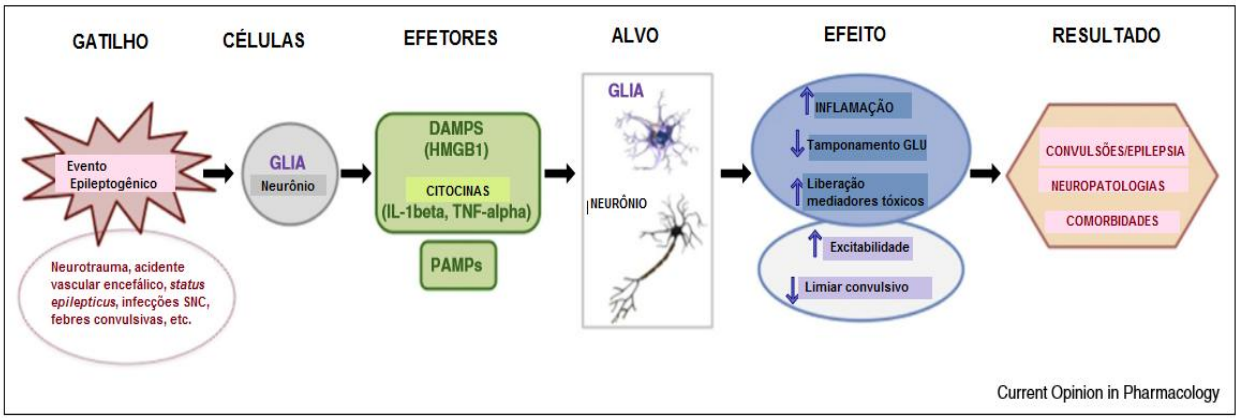
Por outro lado, o GABA é reconhecido como sendo o principal neurotransmissor capaz de gerar potenciais inibidores pré-sinápticos por hiperpolarizar os neurônios. Existem dois tipos de receptores GABA, envolvidos na patogênese da epilepsia, isto é, receptores $GABA_A$ (canais iônicos dependentes de ligantes), que medeiam rápidos potenciais pré-sinápticos inibitórios, por aumentar o influxo de cloreto e receptores $GABA_B$ (receptores acoplados a proteína G) que medeiam lentos potenciais pré-sinápticos inibitórios por aumentar a condutância de potássio e diminuir a entrada de Ca^{2+} (ARONIADOU-ANDERJASKA *et al*., 2008; YOW *et al.,* 2013). Sugere-se então que o sistema gabaérgico pode controlar a indução da epilepsia.

A síntese do GABA é realizada através da enzima glutamato descarboxilase (GAD), incluindo as isoformas GAD67 (GAD 1) que é amplamente difundida nos neurônios gabaérgicos, responsáveis pela atividade neuronal, sinaptogênese e proteção neuronal de uma lesão e GAD65 (GAD2), localizada nas sinapses e terminais nervosos e é responsável pela neurotransmissão gabaérgica. Evidências clínicas tem demonstrado que um erro na transcrição do gene e ou na expressão da proteína GAD contribuem para a patogênese da ELT. A redução da enzima GAD

nos neurônios gabaérgicos podem levar a uma perda de função dos neurotransmissores inibitórios e morte neuronal em focos de epilepsia (WANG *et al* 2016).

Nos últimos anos, cada vez mais se tem apreciado o papel da neuroinflamação e dos mediadores inflamatórios nas desordens do SNC, tornando-se foco de pesquisa atual (Fig. 2). Alguns dados experimentais de epilepsia, já mostraram que há uma relação intrínseca entre inflamação e estresse oxidativo (AGUIAR *et al*., 2012), pois tanto as moléculas específicas inflamatórias, quanto os radicais livres provenientes do estresse oxidativo contribuem para a excitoxicidade e estão envolvidos numa significativa perda de células neuronais após insultos cerebrais, provenientes de crises convulsivas (VEZZANI *et al*., 2016).

Figura 2. Representação esquemática dos eventos moleculares que medeiam a ativação da imunidade inata/inflamação na epilepsia



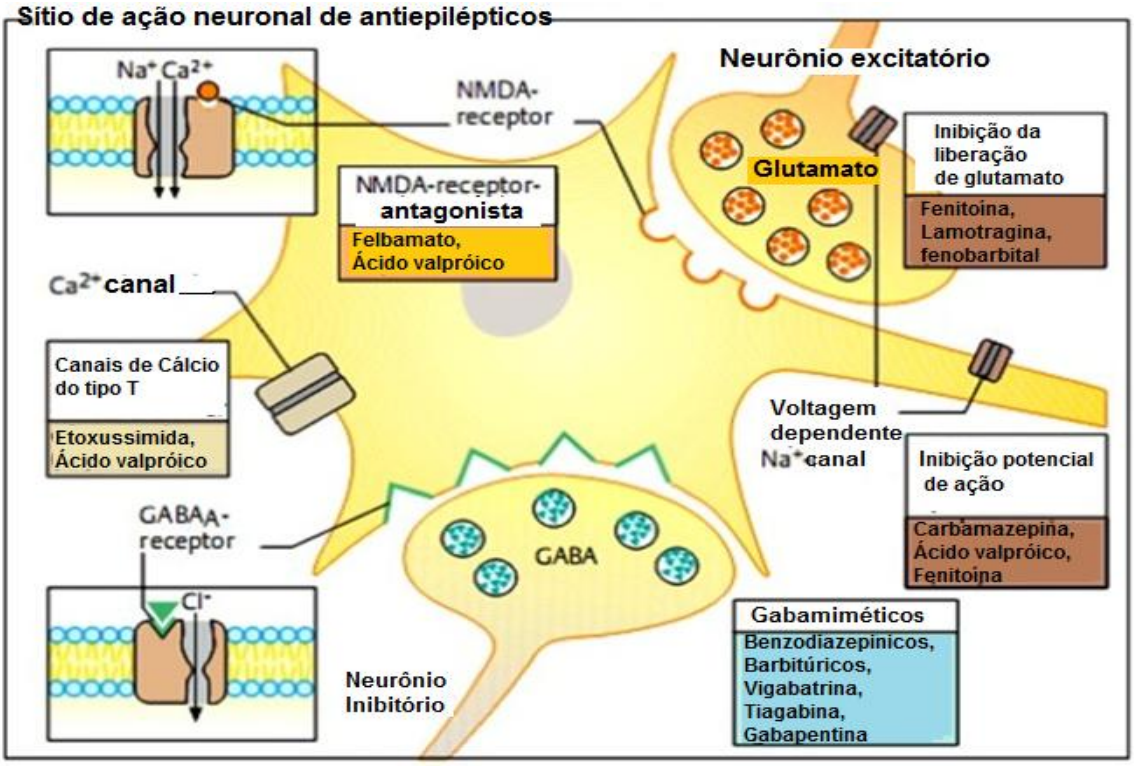
Fonte: Adaptado de IORI, *et al* 2016.

1.1.4 Fármacos anticonvulsivantes

A principal forma de tratamento sintomática para pacientes com epilepsia é o uso de fármacos anticonvulsivantes, também chamados de antiepilépticos, como um possível efeito neuroprotetor (ROGAWSKI *et al*, 2016). Para apresentar atividade anticonvulsivante o fármaco deverá agir em algumas das moléculas do cérebro como os neurotransmissores e seus transportadores, canais de íons e enzimas metabólicas. Embora cada fármaco anticonvulsivante apresente uma característica única, eles podem apresentar múltiplos mecanismos de ação (Fig. 3) (ROGAWSKI *et al*, 2016). Segundo PORTER e colaboradores (2012) podemos dividir os mecanismos de ação em quatro grandes categorias: Estes são (1) Modulação dos

canais de cálcio, sódio e potássio dependentes de voltagem; (2) Alterações na inibição gabaérgica, através de ações sobre receptores GABA ou na síntese, recaptação e degradação do GABA; (3) Diminuição da excitação sináptica através dos receptores de glutamato; (4) Modulação dos neurotransmissores, através de mecanismos pré-sinápticos.

Figura 3. Sítio de ação neuronal de antiepilépticos



Fonte: Adaptado de Lüllmann *et al,*, 2005

Sabe-se que fármacos antiepilépticos possuem tanto efeitos positivos, quanto efeitos negativos no SNC, os grupos de anticonvulsivantes que tem ação no neurotransmissor GABA, incluem os barbitúricos, valproato, vigabatrina e tiagabina, que podem causar fadiga, diminuição cognitiva, e o ganho de peso, além de ter propriedades ansiolíticas e antimaníacos. Já os grupos que tem ação mais específica no neurotransmissor glutamato, incluem o felbamato e lamotrigina que causam perda de peso e possíveis efeitos ansiogênicos e antidepressivos. Topiramato com propriedades tanto gabaérgicas, quanto glutamatérgicas tem um perfil misto. Os barbitúricos, fenobarbital e primidona, têm propriedades conhecidas como psicotrópicas negativas, tais como depressão, hiperatividade, irritabilidade e diminuição da cognição. Carbamazepina, oxcarbazepina, e valproato têm mostrado

resultados positivos como estabilizadores de humor no tratamento de transtorno bipolar e agressão (KAMINSKI *et al*., 2014; HABIBI *et al*., 2016).

Porem esses fármacos utilizados na clínica controlam as crises convulsivas de 70% de pacientes epilépticos, deixando 30% de pacientes refratários aos medicamentos existentes, além de tratar apenas os sintomas das convulsões, não interferindo na sua fisiopatologia (WHO, 2016). Lembrando também que esses fármacos existentes apresentam um grande número de efeitos colaterais, interações farmacocinéticas, baixa tolerabilidade e segurança, altos custo e ainda podem causar dependência (BRODIE *et al*., 2016). Tais resultados podem revelar a necessidade para um melhor entendimento dos mecanismos básicos da epilepsia e posterior desenvolvimento de novas terapias efetivas, com menos efeitos adversos.

1.1.5 Modelos experimentais de Epilepsia

Uma variedade de modelos animais tem sido desenvolvida para estudar e compreender a neurobiologia da condição de epilepsia ou SE. Cada modelo animal demonstra diferentes tipos de epilepsia, ou seja, cada modelo é único e se aproxima mais de um determinado tipo de epilepsia humana (YOW *et al* 2013). Podemos citar como exemplo algumas classes de modelos experimentais de epilepsia, sendo eles modelos elétricos, químicos, térmicos, *in vitro* e ainda o modelo refratário.

O modelo químico envolve a administração de uma substância química convulsivante. Este método é de fácil execução, comparado, por exemplo, com um modelo elétrico, na qual é necessária a implantação de eletrodos. (REDDY E KURUBA, 2013) Diferentes substâncias convulsivantes podem ser administradas para induzir convulsões ou SE em roedores, como por exemplo:

1) Modelo de Pentilenotetrazol (PTZ): É um modelo clássico bem estabelecido para crises epilépticas generalizadas. Sua ação é antagonizar o receptor $GABA_A$, inibindo a ação do GABA (neurotransmissor inibitório) no SNC, induzindo convulsões severas quando administrado em animais. Pode ser usado para desenvolver convulsões agudas, quando administrado uma única vez ou crônica com várias administrações do PTZ no modelo de *kindling* (DHIR, 2012; REDDY E KURUBA 2013).

2) Modelo de Pilocarpina: Descoberto por Tursky em 1983 o modelo de pilocarpina é muito utilizado para induzir SE e tem características muito semelhantes à ELT em humanos (GRONE E BARABAN, 2015) Por ser um agonista colinérgico muscarínico, ativa receptores M1, liberando glutamato e induzindo uma excitabilidade (EYO *et al*., 2016). Alguns estudos constataram a morte celular em regiões como córtex, amígdala, hipocampo, tálamo e substância negra (REDDY E KURUBA 2013).
3) Modelo de 4-AP: Potente convulsivante, sua ação é bloquear correntes de potássio, agindo nas fendas pré-sinápticas o que leva ao aumento da liberação de neurotransmissores, em ambas as sinapses inibitórias e excitatórias, liberando glutamato. Evoca a atividade epileptiforme em modelos animais e *in vitro* (REDDY E KURUBA 2013).
4) Modelo de Aminofilina: As propriedades convulsivas da aminofilina (teofilina-2-etilenodiamina) ainda não estão bem elucidadas, mas até o momento sabe-se que a teofilina bloqueia receptores de adenosina A1 no SNC (STALEY, 2015) o que resulta numa maior excitabilidade e produção de radicais livres, induzindo a convulsões (OCHI, 2011; GULATI, 2005).
5) Modelo de Picrotoxina: Já é bem conhecido que o alcaloide picrotoxina tem ação convulsivante por ser um potente antagonista de receptores neuronais $GABA_A$ bloqueando os canais de cloreto (SALIH E MUSTAFA, 2008; CHINDO *et al*., 2014).
6) Modelo Isoniazida: A isoniazida interfere na síntese do neurotransmissor GABA, inibindo a atividade da enzima GAD, levando a uma rápida depleção de GABA. Esse cenário leva a uma diminuição do sistema inibitório, provocando convulsões (CHINDO *et al*., 2014).

1.2 NEUROINFLAMAÇÃO

A Inflamação é uma sequência de respostas biológicas frente a patógenos e pode ser classificada em aguda ou crônica. Na fase aguda da inflamação, proteínas do plasma e leucócitos dos vasos sanguíneos, extravasam rapidamente para os locais de dano, para iniciar uma série de eventos bioquímicos e celulares envolvendo o sistema imunológico. A inflamação aguda geralmente desaparece em

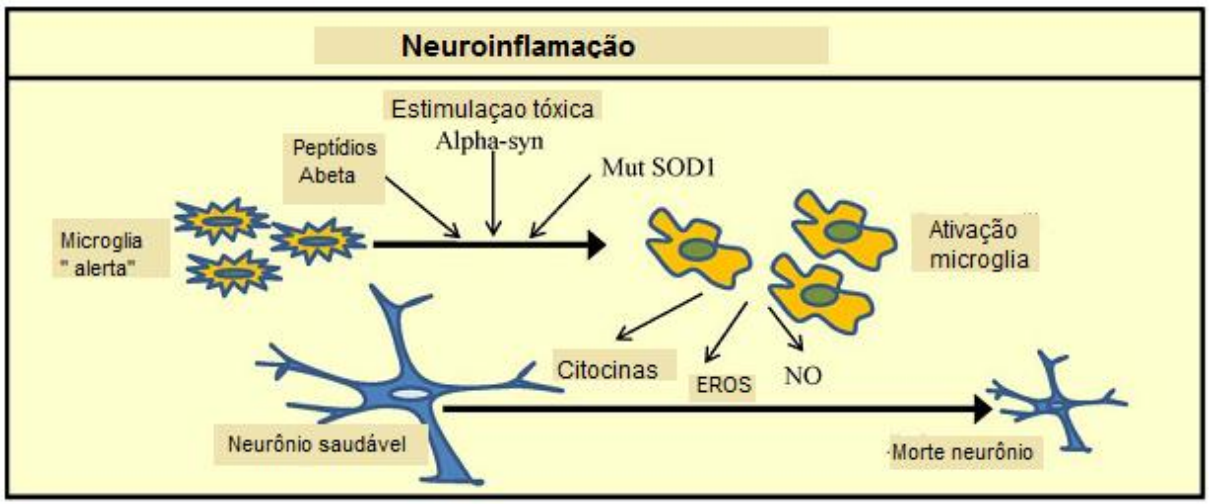
alguns dias, no entanto, em algumas situações a inflamação persiste até virar crônica, aonde então se desencadeia uma rede complexa de vias de sinalizações celulares e moleculares que estão ligadas a varias patologias (VEZZANI *et al*, 2011, DEY *et al*, 2016).

Essas vias de sinalização quando ativadas segregam uma variedade de fatores inflamatórios, incluindo óxido nítrico (NO), citocinas pró-inflamatórias tais como Interleucina-1β (IL-1β), interleucina-6 (IL-6), citocinas anti-inflamatórias como Interleucina-10 (IL-10), espécies reativas de oxigênio (EROS), TNF-α, fatores de crescimento incluindo fator de transformação do crescimento beta (TGF-b) e fator neurotrófico derivado do cérebro (BDNF), que contribuem para a patogênese do dano neuronal em muitas doenças do SNC, incluindo a epilepsia (POLAZZI E MONTI, 2010; GULLO *et al*., 2014).

Em contrapartida, sabe-se que o SNC possui um privilegiado sistema imune de proteção, chamada de barreira hematoencefálica, onde se vê claramente a ativação de astrócitos, células endoteliais e microgliais no processo de reversão da neuroinflamação (Fig. 4) (DEY *et al*, 2016, RASSENDREN E AUDINAT 2016). Alguns dados sugerem que a inflamação, envolvendo células gliais ativadas e aumento da expressão de mediadores inflamatórios específicos, podem contribuir para epileptogênese, devido a alteração da excitabilidade neuronal, favorecendo assim o estabelecimento de uma rede de hiperexcitabilidade neuronal crônica que podem gerar crises espontâneas recorrentes (DEVINSKY *et al*., 2013, WANG *et al*, 2015).

Portanto, compostos com propriedades anti-inflamatórias ou com capacidade para inibir a ativação microglial podem representar uma promissora estratégia para melhorar os efeitos terapêuticos sobre epilepsia (WANG *et al*, 2015) e ainda tornar a neuroinflamação como um potente biomarcador mecanicista para a epileptogênese e ictiogênese, favorecendo a descoberta de novas vias para o tratamento da epilepsia (PITKÄNEN *et al*, 2016).

Figura 4. Hipóteses sobre o papel microglial em doenças neurodegenerativas



Fonte: Adaptado de: POLAZZI E MONTI, 2010.

1.3 ESTRESSE OXIDATIVO

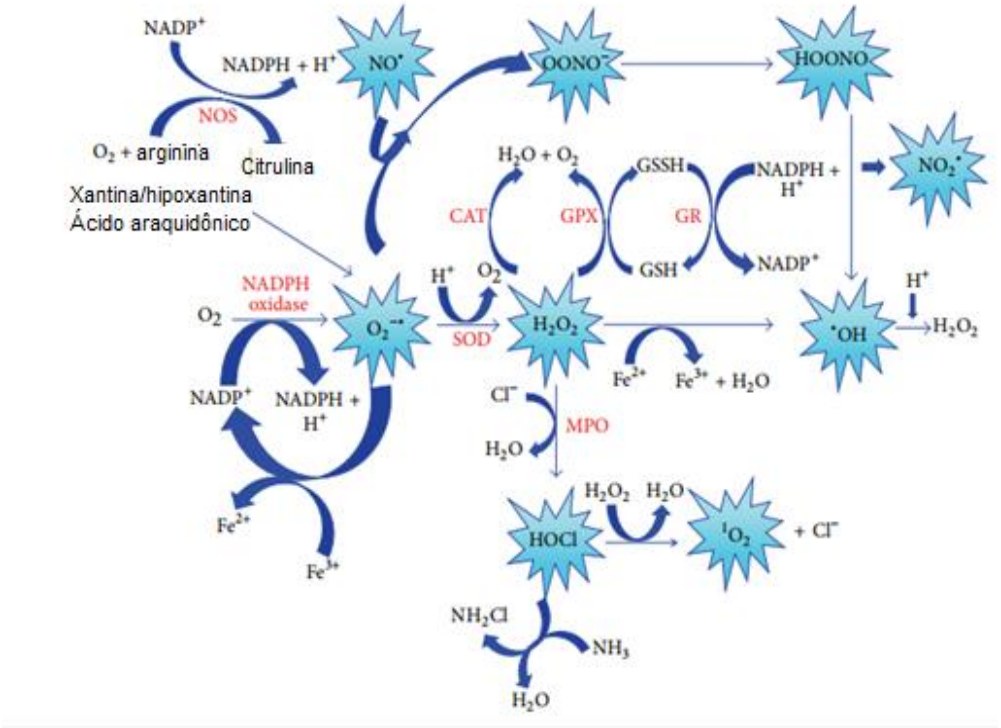
Como mencionado anteriormente, além da influência da inflamação, outro mecanismo envolvido diretamente na patogênese da epilepsia é o estresse oxidativo, pois tanto as moléculas específicas inflamatórias quanto os radicais livres provenientes do estresse oxidativo estão envolvidos numa significativa perda de células neuronais após insultos cerebrais, provenientes de crises convulsivas (AGUIAR *et al*., 2012; VEZZANI *et al*., 2016).

O estresse oxidativo foi descrito como um desequilíbrio entre geração e eliminação de EROS e espécies reativas de nitrogênio (ERNS) e ainda alterações na sinalização do NO. Radicais livres consistem em estruturas químicas com um ou mais elétrons desemparelhados. A produção em excesso de radicais livres está associada com danos causados às estruturas celulares, incluindo a peroxidação lipídica, danos no DNA, e a inativação de enzimas. O SNC torna-se altamente sensível devido ao seu elevado consumo de oxigênio e baixa atividade das defesas antioxidantes, resultando na patogênese das condições do SNC, tais como epilepsia (DAL-PIZZOL *et al*., 2000; AGUIAR *et al*., 2012).

Os radicais provenientes do oxigênio, são as espécies reativas geradas mais importantes dos sistemas vivos (Fig. 5). Incluem o radical superóxido ($O_2^{\cdot-}$), radical peroxil ($ROO^{\cdot}$), radical hidroperoxil ($HO_2^{\cdot}$), radical hidroxila ($OH^{\cdot}$), e espécies de radicais sem elétrons livres tais como, peróxido de hidrogénio (H_2O_2) e o oxigênio

singlete ($^1O_2$), que são facilmente convertidos em radicais livres. O $NO^{\cdot}$, dióxido de nitrogênio ($NO_2^{\cdot}$), e peroxinitrito ($OONO^-$) representam as mais importantes ERNS (QUIÑONEZ-FLORES *et al*., 2016).

Figura 5. Geração de espécies reativas de oxigênio e nitrogênio (EROS/ERNS)



Fonte: Adaptado de: QUIÑONEZ-FLORES *et al*, 2016.

Evidências demonstram que outro fator que já está bem estabelecido na patogênese da epilepsia, juntamente com o estresse oxidativo, são as disfunções mitocondriais (FOLBERGROVÁ *et al*., 2016). A mitocôndria produz energia na forma de adenosina trifosfato (ATP) e tem um papel fundamental na homeostase de Ca^{2+} intracelular, biossíntese de neurotransmissores, mecanismo de morte celular, geração de EROS estimulando consequentemente a excitoxicidade (MARTINC *et al*., 2012).

Antioxidante é definido como qualquer agente que em baixas concentrações retarda ou inibe a velocidade de oxidação de uma substância oxidável (HALLIWELL e GUTTERIDGE, 2007). Os danos induzidos pelo estresse oxidativo podem ser minimizados pelo sistema de defesa antioxidante. As defesas antioxidantes não enzimáticas incluem antioxidantes lipofílicos (tocoferóis, carotenóides, e bioflavonóides) e hidrofílicos (glutationa e ascorbato). O sistema de defesa

antioxidante enzimático, está representado pelas enzimas SOD, CAT, glutationa-peroxidase (GPx), glutationa redutase (GR) e a glutationa-S-transferase (GST). Essas enzimas evitam o acumulo de $O_2^{\cdot -}$, H_2O_2 e a consequente produção de $OH^{\cdot}$. A SOD representa o mecanismo de defesa central dos organismos vivos, por catalisar a formação de H_2O_2 a partir de radicais $O_2^{\cdot -}$ e a enzima tetramérica CAT contem grupos heme que degradam H_2O_2 (BORELLA e VARELA, 2004; HWANG e KIM, 2007; KARIHTALA e SOINI, 2007; QUIÑONEZ-FLORES *et al*., 2016).

É crescente o número de investigações referentes a antioxidantes naturais ou sintéticos, como uma estratégia terapêutica eficiente, na prevenção de patologias associadas ao estresse oxidativo, como a própria epilepsia, uma vez que a relação entre estresse oxidativo e doenças que afetam o SNC está bem estabelecida (CÁRNENAS-RODRIGUEZ *et al*., 2013). Pesquisas sugerem que o aumento na quantidade de radicais livres e/ou diminuição na atividade de defesas antioxidantes podem ser reportados durante os processos convulsivos (NAFFAH-MAZZACORATTI *et al*., 2001).

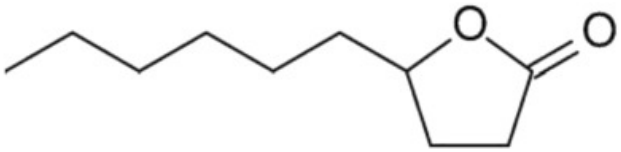
1.4 GAMA-DECANOLACTONA

Aeollanthus suaveolens, é uma planta nativa da Amazônia, que pertence à família Lamiaceae e é conhecida popularmente como catinga-de-mulata, ou macassá. A planta é usada pela população em banhos de cheiro feito pela infusão de plantas aromáticas, em motivos religiosos ou folclóricos. Na etnomedicina é usado por caboclos da Amazônia, por suas alegadas características medicinais (sedativa, anticonvulsivante, analgésica, hipnótica e ansiolítica) sendo a folha a parte mais utilizada na preparação do chá e sumo. Estudos anteriores mostraram que o óleo essencial das folhas e flores obtido por hidrodestilação apresenta como principais componentes o (-) linalol, farneseno e (-) massoia lactona (ELISABETSKY *et al*., 1995; OLIVEIRA *et al*., 2004). Estudos fitoquímicos monitorados farmacologicamente evidenciaram que o óleo essencial é o responsável pelo bloqueio de convulsões induzidas por PTZ em camundongos. A atividade anticonvulsivante das lactonas detectadas nesta planta, (δ-decalactona, δ-decen-2-lactona) e γ-decanolactona, uma lactona com estrutura semelhante, porém sintetizada, foram avaliadas em camundongos, sendo que as duas primeiras não

apresentaram resultados significativos, entretanto a ɣ-decalactona sintética apresentou atividade sedativa (COELHO *et al*., 1996).

Gama-decanolactona (GD) (Fig. 6) é um composto monoterpênico com estrutura química semelhante às lactonas presentes no óleo essencial de *Aeollanthus suaveolens* (ELISABETSKY *et al*., 1995; COELHO DE SOUZA *et al*., 1997; PFLÜGER *et al.*, 2016).

Figura 6. Estrutura química Gama-decanolactona (GD)



Fonte: Pflüger *et al.*, 2016.

Estudos anteriores mostraram que GD, quando testada em camundongos, apresenta atividade hipnótica, anticonvulsivante e hipotérmica (COELHO DE SOUZA *et al*., 1997). Quando testada nos modelos experimentais do nado forçado e do labirinto em cruz elevada, GD não demonstrou efeito, sugerindo não apresentar atividade do tipo antidepressiva e ansiolítica (VIANA *et al*., 2007). No modelo de *kindling*, considerado um importante modelo de epileptogênese, apresentou significativo efeito protetor em convulsões induzidas por PTZ e também demonstrou atividade antigenotóxica, uma vez que reparou o dano ao DNA, induzido por PTZ (OLIVEIRA *et al*., 2008). Ainda, a avaliação neuroquímica realizada através de ensaio de *binding*, mostrou que GD foi capaz de inibir de forma dose dependente a união específica de L-[3H]-glutamato, podendo esta ação estar envolvida na atividade anticonvulsivante deste composto (PEREIRA *et al*., 1997).

Alguns resultados *in vitro,* recentemente demonstraram que a GD, atenuou a ativação de células microgliais N9, no modelo de LPS que é uma endotoxina, presente em bactérias gram-negativas. Concomitantemente GD demonstrou diminuir a expressão de marcadores inflamatórios como a enzima iNOS, TNF-α e consequentemente inibir a formação de EROS induzidas por LPS nas células microgliais. Além disso, GD bloqueou marcadores de apoptose como a fosforilação da p38 e ativação da caspase-9 clivada, diminuindo o dano causado ao DNA (PFLÜGER *et al*., 2016). Estes dados indicam que GD possui um potencial

terapêutico neuroprotetor, e que exerce os seus efeitos através da inibição da inflamação.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o perfil farmacológico de Gama-decanolactona em diferentes modelos de epilepsia, bem como, sobre parâmetros de estresse oxidativo e neuroinflamação.

2.2 OBJETIVOS ESPECÍFICOS

Avaliar o efeito de Gama-decanolactona *in vitro*:

- Sobre a viabilidade celular, produção de espécies reativas de oxigênio, citocinas pró-inflamatórias, genotoxicidade e morte celular em linhagem de células microgliais N9.

Avaliar o efeito de Gama-decanolactona *in vivo*:

- Sobre convulsões induzidas por pilocarpina e sobre produção intracelular das espécies reativas de oxigênio, enzimas antioxidantes, parâmetros inflamatórios e genotoxicidade em córtex de camundongos.

- Sobre convulsões induzidas por diferentes agentes: isoniazida, picrotoxina, 4-aminopiridina e aminofilina;

- no modelo da barra giratória (*rotarod*);

- no sono induzido por diazepam;

3 ARTIGO CIENTÍFICO I

Gamma-decanolactone inhibits iNOS and TNF-alpha production by lipopolysaccharide-activated microglia in N9 cells.

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Immunopharmacology and inflammation

Gamma-decanolactone inhibits iNOS and TNF-alpha production by lipopolysaccharide-activated microglia in N9 cells

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ABSTRACT

Activated microglia that produce reactive nitrogen species (RNS), inflammatory factors, reactive oxygen species (ROS), and other neurovirulent factors may lead to the development of neurodegenerative diseases. Certain compounds can inhibit the activation of microglia. However, these mechanisms remain unclear. In the present study, we investigated the inhibitory effect of Gamma-decanolactone (GD) on the production of reactive oxygen species and inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS) - stimulated N9 murine microglial cells through the p38 MAPK signaling pathway. The results showed that GD attenuated the activation of N9 cells and inhibited intracellular reactive oxygen species and the expression of iNOS and TNF-α induced by LPS in the cells. In addition, GD blocked the phosphorylation of p38 and inhibited cleaved caspase-9 and DNA damage. These data indicate that GD has therapeutic potential for the treatment of neurodegenerative diseases, and that it exerts its effects by inhibiting inflammation.

1. Introduction

Microglia, differentiated from the active phagocytic amoeboid monocytes (Hickey and Kimura, 1988; Rock et al., 2004), are brain resident immunocompetent cells originating from bone marrow-derived monocytes that invade the brain during embryogenesis. Activated microglia produce inflammatory and neurovirulent factors like reactive nitrogen species and reactive oxygen species have cause neuron injury, ultimately resulting in neurodegenerative diseases such as epilepsy (Palace, 2007; Ojo et al., 2015; Fakhoury, 2015). Several factors have been identified and are known to provoke microglial activation. Lipopolysaccharide (LPS), the major portion of the outer membrane of Gram-negative bacteria, is regarded as the main risk factor responsible for microglial activation (Kim and Joh, 2012).

Among the LPS-induced reactions, the overproduction of nitric oxide (NO) generated by NO synthase (NOS) has received much attention due to the role played in microglial activation. Inducible NOS (iNOS) is the most important NOS involved in this process. In a physiological microenvironment, NO causes vasoconstriction, acts as a neuroendocrine mediator in the central nervous system (CNS), and has protective functions in anti-inflammatory pathways. Elevated NO levels may also be beneficial in response to immunological stimuli as a defense mechanism against microbial or viral insults. Additionally, NO can also be produced in response to factors resulting from chronic inflammatory conditions, such as epilepsy (Aguiar et al., 2012; Mula, 2015).

Epilepsy is a disorder characterized by aberrant synchronous excitation of brain regions that disrupt normal functioning and that causes specific signs and symptoms, including recurrent seizures (Da Fonseca et al., 2014; Coelho et al., 2015). Experimental data have demonstrated the intrinsic relationship between epilepsy, oxidative stress, and inflammation, and showed that specific inflammatory molecules such as free radicals generated by oxidative stress are involved in a significant loss of neuronal cells after cerebral insults from seizures (Aguiar et al., 2012; Devinsky et al., 2013).

Gamma-decanolactone (GD) (Fig. 1) is a monoterpene compound. A psychopharmacological study using mice revealed that GD has a dose-dependent effect on the central nervous system, with hypnotic, anticonvulsant, and hypothermic activities (De Oliveira et al., 2008). Previous in vivo and *ex vivo* research revealed the protective effect of GD on seizures induced by pentylenetetrazol, with a single 0.3 g/kg GD dose causing lesser DNA damage in the brain tissue of the PTZ-kindled mice. The results suggested protective effects associated with detected anticonvulsant activity,

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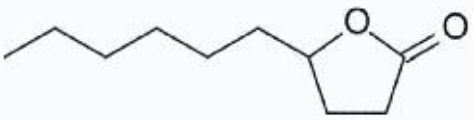


Fig. 1. Chemical structure of Gamma-decanolactone (GD).

and indicate that GD is able to inhibit the glutamate binding in cortex homogenate (Pereira et al., 1997; Viana et al., 2007; De Oliveira et al., 2008).

Based on the protective effect on seizures of GD, the present study investigated the potential and possible mechanism through which this drug inhibits microglia activation by proinflammatory stimulants by LPS in N9 murine microglial cell line.

2. Materials and methods

2.1. Chemicals

Roswell Park Memorial Institute (RPMI) 1640 Medium, low-melting-point agarose (LMP), high-melting-point agarose (HMP), phosphate-buffered saline (PBS; Na_2HPO_4, KH_2PO_4 and KCl, pH 7.4) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from Gibco-BRL (Grand Island, NY, USA). Primary antibody (anti-p38 [Sc7149], anti-p-p38 [Sc101759], anti-iNOS [Sc7271], anti-TNF-α [Sc52746], and anti-β-actin [Sc69879]) and secondary antibody (anti-rabbit and anti-mouse) were obtained from Santa Cruz Biotechnology (California, USA). DPPH (1, 1-diphenyl, 2-picrylhydrazyl) and vitamin E were obtained from Sigma Chemical Co. USA. All other reagents were of analytical grade.

2.2. Culture conditions

The N9 murine microglial cell line was a gift from Dr[a]. Teresa Faria Pais (University of Lisbon, Lisbon). N9 Cell lines were grown in RPMI supplemented with 10% FBS, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The N9 microglial cells were plated into 6-well plates in all experiments. When subconfluence was reached, cells were pre-treated for 20 h with RPMI culture medium containing GD (10, 50, and 100 μM). The cells were then exposed to LPS (1 μg/ml) diluted in culture medium for 4 h at 37 °C after washing twice with phosphate-buffered saline (PBS, pH 7.4). For all experiments, the negative control was the N9 cells with 5% Tween and the positive control was the N9 cells induced with LPS only. For cell treatments, a stock solution of GD was prepared freshly prior to use, with Tween 5% as solvent. The appropriate concentrations were obtained by diluting the stock solution in sterile distilled water.

2.3. Trypan blue dye exclusion assay (TBDE)

The trypan blue dye exclusion assay (TBDE) was used to determine relative cell viability based on cytotoxic measurements. For each group, 10 μl cell suspension (1×10^4 cells/ml) was mixed with 10 μl trypan-blue 0.4% solution (Viau et al., 2009). Cytotoxicity (the cellular growth inhibitory rate) was determined from the number of viable cells (no color) in treated samples as a percentage of the PBS control using the Countess® Automated Cell Counter (Invitrogen). The test was carried out according to the manufacturer's instructions.

2.4. Reactive oxygen species detection by flow cytometric analysis

Levels of intracellular reactive oxygen species were estimated following treatment with GD using 2',7'-dichlorofluorescein diacetate (H_2DCFDA, Sigma) as a fluorescent probe. Oxidative stress was detected by incubating the cells (1×10^4 cells/ml) with H_2DCFDA 20 μM for 30 min at 37 °C. Cells were then detached by trypsinization and washed twice with PBS. After filtration through a cell strainer cap, cells were analyzed using a FACS Calibur flow cytometer with CellQuest software in accordance with Bass et al. (1983). A total of 10,000 events were measured per sample. DCF fluorescence intensity was shown in arbitrary units.

2.5. Alkaline comet assay

The alkaline comet assay was performed as previously described by Singh et al. (1988). Briefly, 10 μl cell suspension (1×10^4 cells/ml) treated with GD was mixed with 90 μl LMP agarose, spread on a normal agarose precoated microscope slide, and placed at 4 °C for 5 min to allow for solidification. Cells were lysed in a high-concentration salt and detergent solution (NaCl2.5 M, Na_2EDTA100 mM, Tris 10 mM with Triton X-100 1%, and DMSO 10% freshly added) for 2 h. Slides were removed from lysing solution and washed three times with PBS. Subsequently, cells were exposed to alkali conditions (NaOH 300 mM/ Na_2EDTA1 mM, pH > 13, 30 min, 4 °C) to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 25 min at 25 V and 300 mA (94 V/cm). After electrophoresis, the slides were neutralized and silver stained (Nadin et al., 2001). One hundred cells were scored visually according to tail length and the amount of DNA present in the tail. Each comet was given an arbitrary value of 0–4 (0, undamaged; 4, maximally damaged), as described by Collins et al. (1995). Damage score was thus assigned to each sample, and ranges from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4). International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method, as it is highly correlated with computer-based image analysis (Collins et al., 1995; Burlinson et al., 2007).

2.6. Quantification of cleaved caspase-9 by flow cytometric analysis

After treatment, cells (1×10^6 cells/ml) were harvested, resuspended in 25 μl PBS and fixed with formaldehyde 4%. After permeabilization and blocking (Triton X-100 0.2% in PBS and BSA 1%), cells were incubated with *anti*-caspase-9 antibody (diluted 1:1000) for 1 h at room temperature, followed by incubation with anti-rabbit FITC secondary antibody (Uniscience) (diluted 1:1000) for 1 h at room temperature in the dark. A total of 10,000 events were analyzed per sample by FACS Calibur flow cytometer. Fluorescence intensity in arbitrary units was plotted in histograms; the mean fluorescence intensity was calculated using CellQuest software (Viau et al., 2014).

2.7. Western blotting

The N9 cells (1×10^7 cells/ml) were treated with GD for 20 h and then exposed to LPS, after which they were washed twice with PBS and resuspended in lysis buffer (Tris/acetate 20 mM, pH 7.5, sucrose, EDTA 1 mM, EGTA 1 mM, Triton X-100 1%, ortovanadate 1 mM, sodium glycerophosphate 1 mM, sodium fluoride 5 mM, sodium pyrophosphate 1 mM, β-mercaptoethanol 5 mM, bezamidine 1 mM, PMSF 35 μg/ml, leupeptine 5 μg/ml). The samples were homogenized and incubated on ice for 20 min before being centrifuged at 12,000g for 15 min. The supernatants were assayed for protein concentration.

For western-blot analyses, a 30 µg sample of total proteins was separated on SDS-polyacrylamide gel 15% and transferred to a polyvinylidene difluoride membrane. The membranes were blocked by incubation in TBS buffer containing Tween-20 0.1%, and dried milk 5% for 1 h at room temperature, and then washed with TBS buffer containing Tween-20 0.1%. Next, membranes were blotted overnight at 4 °C with the primary antibodies (1/1000) mentioned above. The blots were washed three times with TBS 0.1% Tween and developed with peroxidase-linked secondary antibodies (1/3000). All blots were developed by ECL Western Blotting Detection Kit Reagent and detected using a ChemiDoc (Bio-Rad) imaging system. In recovery experiments, cells were pre-treated with the antioxidant NAC as described above.

2.8. DPPH radical scavenging method

The radical scavenging activities of the compounds were determined as previously described (Di Mambro et al., 2003). GD and vitamin E were tested at 1, 5, 10, 50, 75, 100, 250, and 500 µM. Vitamin E was used as positive control. DPPH (diluted in methanol) was added to the final concentration of 0.5 mM and allowed to react at room temperature for 30 min in the dark. Absorbance was measured at 517 nm using Spectra Max Plate Reader® M² (Molecular Devices), Sunnyvale, California, USA. Percent scavenging of the DPPH free radical was measured using the following equation: % Scavenging Activity = [(Absorbance of the control − Absorbance of the test sample)/Absorbance of the control] × 100.

2.9. Statistical analysis

All experiments were independently repeated at least three times, with triplicate samples for each treatment. Results are expressed as means ± standard deviation (S.D.). Data were analyzed by one-way analysis of variance (ANOVA), and means were compared using Tukey test, with $P \leq 0.05$ considered as statistically significant, using GraphPad Prism v5 program (Intuitive Software for Science, San Diego, CA, USA).

3. Results

3.1. GD and LPS treatment display no inhibitory effect on N9 microglial cells

The trypan blue assay was used to evaluate whether the administered dose of GD or LPS influenced N9 cell viability. The results showed that incubation with LPS (10, 100, and 1000 ng/ml) alone for 24 h had no effect on the viability of N9 cells (Fig. 2A). Additionally, treatment with GD (10, 50, and 100 µM) for 24 h showed no inhibitory effect on N9 cell viability in comparison with the vehicle-treated group (Fig. 2B). Thus, the effect of GD in preventing microglial activation was not caused by the cytotoxicity of reagents in previous experiments.

3.2. Inhibitory effect of GD on intracellular reactive oxygen species formation induced by LPS in N9 microglial cells

The production of reactive oxygen species is a risk factor in microglial activation induced by inflammatory factors. Therefore, we assessed intracellular reactive oxygen species induced by LPS in N9 cells by monitoring the changes in fluorescence intensity of the probe, DCFH-DA (Fig. 3A-B). The results indicated that the amount of cells with fluorescence induced by 1 µg/ml LPS for 4 h significantly increased compared to the vehicle-treated control group. By contrast, pre-treatment with GD for 20 h before exposure to LPS suppressed the production of DCFH fluorescence in a

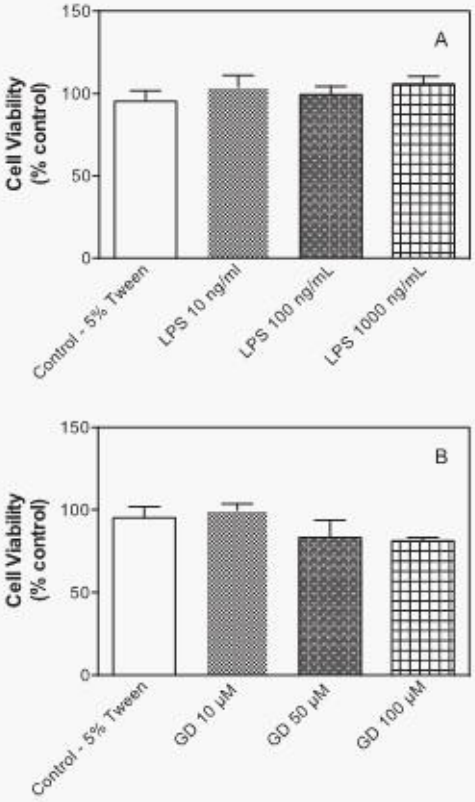


Fig. 2. Effect of GD and LPS on N9 cell viability evaluated by the trypan blue assay. (A) N9 cells were treated with LPS (10–1000 ng/ml) and incubated for 24 h. (B) N9 cells were treated with GD (10, 50, and 100 µM) for 20 h. Values represent the mean ± S.D. of three independent experiments.

dose-dependent manner. The suppressive effect of GD in both doses (50 and 100 µM) was considered to be significant, compared to the group treated with LPS alone ($P < 0.01$ and $P < 0.001$, respectively). These results suggest that one of the reasons that GD suppresses microglial activation is the decrease in the production of reactive oxygen species.

3.3. Inhibitory effect of GD on genotoxicity formation induced by LPS in N9 microglial cells

The alkaline (pH > 13) comet assay detects DNA strand breaks and alkali-labile sites. Our results showed that LPS induces significant DNA damage, and that both GD doses (50 and 100 µM) abrogate the generation of this damage (Fig. 4).

3.4. Inibitory effect of GD on cleaved caspase 9 activity induced by LPS in N9 microglial cells

We analyzed the cleavage and consequent activation of caspase-9 by flow cytometry using specific antibodies that recognize only the intact (inactive) forms of the enzyme. The results,

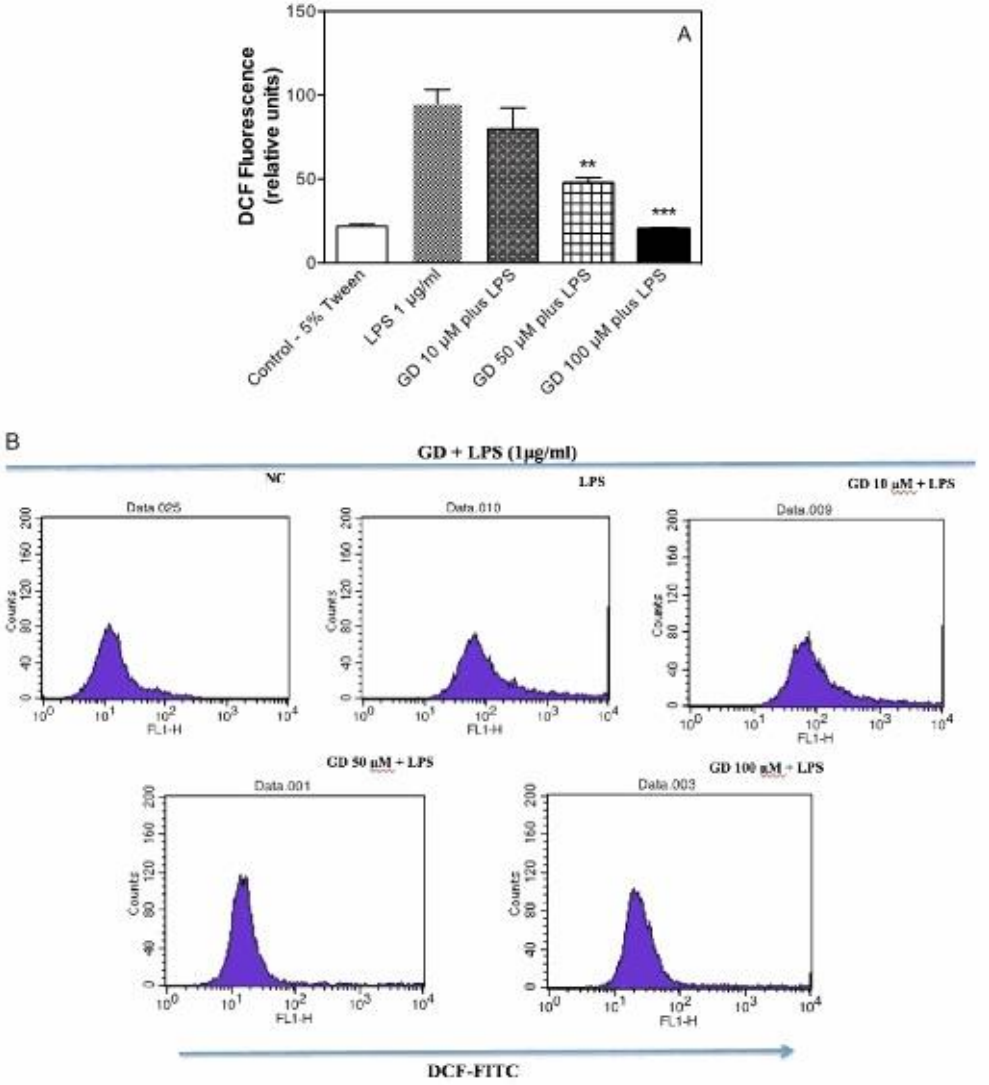


Fig. 3. (A) Inhibitory effect of GD on the intracellular reactive oxygen species formation in N9 cells. Cells were pre-treated with GD (10, 50, and 100 μM) for 20 h and exposed to LPS (1 μg/ml) for 4 h. Intracellular reactive oxygen species were assessed by monitoring the change in fluorescence intensity. Values represent the mean ± S.D. of three independent experiments. **P < 0.01, ***P < 0.001 compared to the group treated with LPS alone. (B) Representative histograms. FL1-H: relative DCF fluorescence intensity.

summarized in Fig. 5A-B, indicate that there is a clear difference on the induction of cleaved caspase-9 when the N9 cells are treated with GD 50 and 100 μM. Moreover, the lower GD dose (10 μM) caused no significant changes in the levels of cleaved caspase-9 in N9 cells (Fig. 5A-B). These results were consistent with the data from reactive oxygen species analysis and genotoxicity assay; in fact, the treatment with GD 50 μM and 100 μM induced a stronger protective effect on N9 cells induced by LPS, when compared to LPS alone (Figs. 3A-B and 4).

3.5. *Effect of GD on iNOS and TNF-α production induced by LPS in N9 microglial cells*

iNOS plays an important role in generating the production of

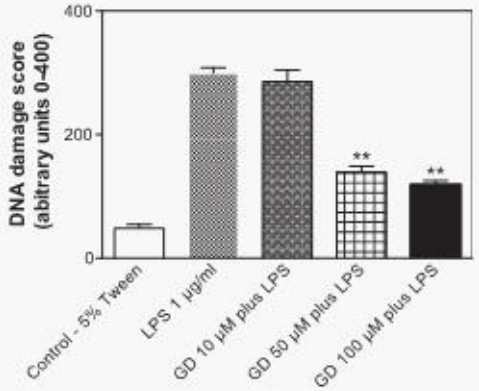


Fig. 4. Effect of GD on DNA damage index in N9 cells. Cells were pre-treated with GD (10, 50, and 100 µM) for 20 h and exposed to LPS (1 µg/ml) for 4 h. Values represent the mean ± S.D. of three independent experiments. $^{**}P < 0.01$ compared to the group treated with LPS alone.

NO in cells. The expression of iNOS is usually high in neuron cells induced by inflammatory factors, such as LPS. TNF-α is the major neurotoxic agent secreted by β-amyloid (Aβ-stimulated microglia, Combs et al., 2001), which can also cause neuronal cell death both directly and indirectly via the induction of NO and reactive oxygen species in microglial cells (Hu et al., 1997). The increased levels of the iNOS and TNF-α proteins induced by LPS were reduced by GD pre-treatment in a dose-dependent manner (Fig. 6). Our results demonstrate that the increasing expression of iNOS and TNF-α in N9 cells induced by LPS was inhibited by the GD.

3.6. *Effect of GD on the phosphorylation of p-38 mitogen-activated protein kinase (MAPK) induced by LPS in N9 microglial cells*

To investigate whether GD influences underlying pathways followed by the upregulation of reactive oxygen species and iNOS levels induced by LPS in microglial cells, we examined the effect of GD on LPS-induced p-p38 activation in N9 cells. The LPS-induced phosphorylation of p38 (Fig. 6) was inhibited by GD. These findings suggested that the phosphorylation of p38 involved in the production of reactive oxygen species and iNOS induced by LPS was downregulated by GD.

These results also indicated that GD abolishes reactive oxygen species generation, DNA damage, and induction of apoptosis in N9 cells induced by LPS, therefore contributing to neuroprotection.

3.7. *GD did not present any scavenger effects on DPPH radicals*

The change in DPPH is relatively stable nitrogen centered free radical that can easily accept an electron or hydrogen radical to form a more stable diamagnetic molecule (Mai et al., 2012). The DPPH radical scavenging activity of GD is given in Fig. 7. GD did not present any scavenger effects on DPPH radicals. The DPPH antioxidant assay is developed based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for a visible deep purple color of DPPH in alcoholic solution and the color intensity can be measured as absorbance at 517 nm. When DPPH accepts an electron donated by an antioxidant compound, DPPH is decolorized, which can be quantitatively measured from the changes in absorbance (Uddin et al., 2014).

4. Discussion

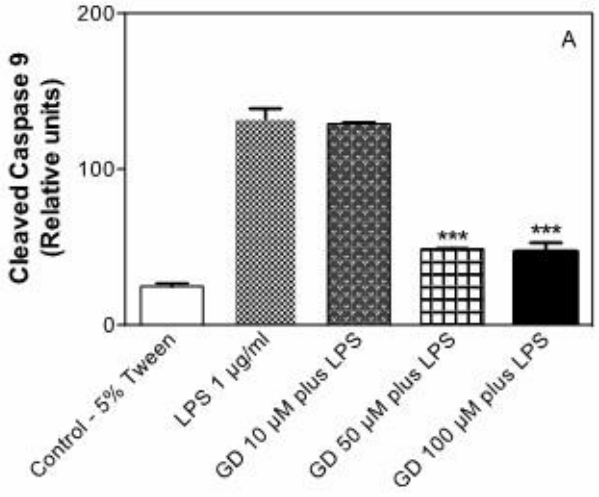
It is well known that activated microglia produce NO and pro-inflammatory cytokines, such as TNF-α, which alter their cellular functions and initiate an inflammatory cascade associated with several neurodegenerative diseases (González-Scarano and Baltuch, 1999; Ziebell et al., 2015). To the best of our knowledge, our results demonstrate, for the first time, that GD blocks the underlying pathways of p38 MPK followed by the decrease in reactive oxygen species, iNOS, and TNF-α levels in LPS-treated microglial cells. Our data suggest that GD may be beneficial for the treatment of neurodegenerative diseases.

NO is accepted as an active messenger molecule in the cardiovascular and nervous systems (Toda et al., 2009; Mattila and Thomas, 2014), and is formed endogenously by the conversion of L-arginine to L-citrulline by NOS (Mayer et al., 1989). It has been shown that activated microglial cells kill neurons via NO from iNOS by inhibiting neuronal respiration (Bal-Price and Brown, 2001; Brown and Bal-Price, 2003). We found that pre-treatment with GD suppressed iNOS and TNF-α levels in LPS-treated N9 cells. This is one of the proposed mechanisms by which GD prevents microglial activation induced by LPS.

Reactive oxygen species are normal metabolites of oxidation-reduction reaction in cells, whereas overproduction of reactive oxygen species induces a series of physiopathological events (Zalba et al., 2007). Based on this fact, the suppression of reactive oxygen species may be an effective way to protect cells from inflammatory damage. Therefore, we examined intracellular reactive oxygen species formation in N9 cells. Our findings clearly show that GD suppressed the production of reactive oxygen species in a dose-dependent manner. There was no significant cytotoxic effect on cell viability when N9 cells were incubated with GD (10, 50, and 100 µM) for 24 h, indicating that the downregulation of iNOS and reactive oxygen species levels by GD was not due to a decrease in cell numbers. In addition, the effect of antioxidant compounds on DPPH radical scavenging is involved with their potential to donate a hydrogen atom. Thus, in the present study, we also demonstrate that GD did not present any scavenger effects on DPPH radicals, suggesting that the antioxidant mechanism of action of GD may not be related to their ability to donate an electron or hydrogen radical.

We showed that GD blocked the phosphorylation of p38 MAPK. It has been well documented that p38 MAPK initiates mitochondrial apoptotic pathway through both enhancing transcription of proapoptotic genes and directly activating them (Sui et al., 2014). Release of cytochrome c and other proapoptotic molecules that initiate the apoptotic cascade, followed by the cleavage of caspase-9 in the hippocampus, has been observed after seizure induced by intraamygdaloid injection of kainic acid. Moreover, increasing evidence has emerged indicating that apoptosis plays an important role in cell death after various pathological brain insults (Chuang et al., 2007). So, the inhibitory effect of GD on cleaved caspase 9 activity in N9 cells may be associated with the already known anticonvulsant activity of GD in mice.

The intrinsic pathway is triggered following disruption to intracellular organelle homoeostasis or DNA damage (Henshall, 2007). Several triggers for this pathway have been identified, including raised intracellular calcium, free radicals and dimerization interactions of Bcl-2 family proteins, as well as pro- and anti-apoptotic regulators of apoptosis that regulate mitochondrial and other intracellular organelle (dys)function (Henshall and Simon, 2005). Mitochondrial dysfunction culminates in the release of apoptogenic factors such as cytochrome *c*, which activates Apaf-1 (apoptotic protease-activating factor 1) and caspase 9, followed by downstream executioner caspases. Caspase-independent mechanisms have also been identified including AIF (apoptosis-



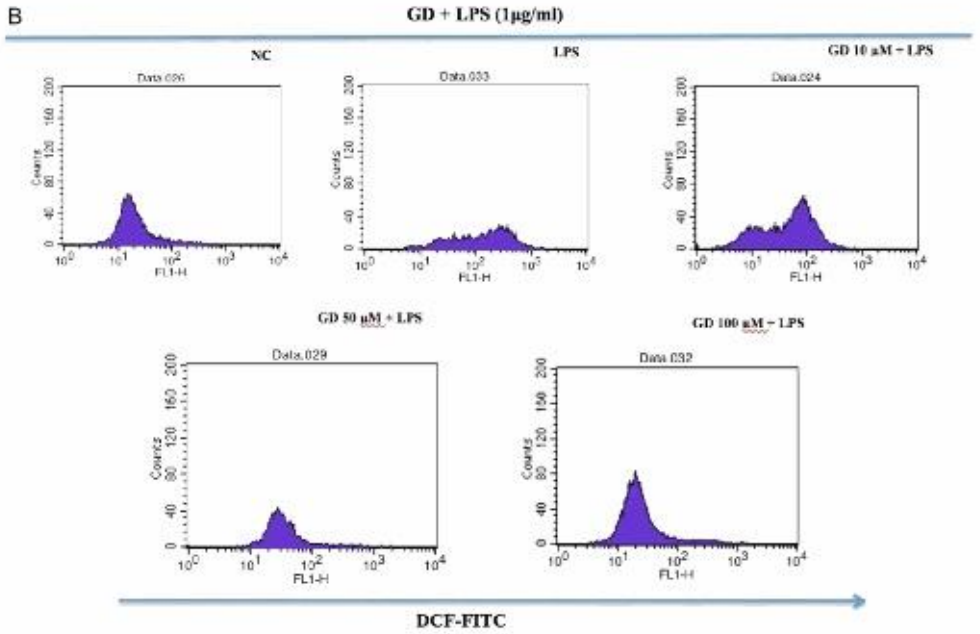


Fig. 5. (A) Inhibitory effect of GD on cleaved caspase 9 activity in N9 cells. Cells were pre-treated with GD (10, 50, and 100 µM) for 20 h and exposed to LPS (1 µg/ml) for 4 h. Cleaved caspase 9 activity was assessed by monitoring the change in fluorescence intensity. Values represent the mean ± S.D. of three independent experiments. ***P < 0.001 compared to the group treated with LPS alone. (B) Representative histograms. FL1-H: relative cleaved caspase 9 activity intensity.

inducing factor), a mitochondrial protein that translocates to the nucleus to induce large-scale DNA fragmentation and apoptosis (Henshall and Murphy, 2008).

In the present study, we targeted the p38 MAPK signaling pathway as a novel approach to the management of epilepsy (Fig. 8). GD inhibited microglial activation, attenuated reactive

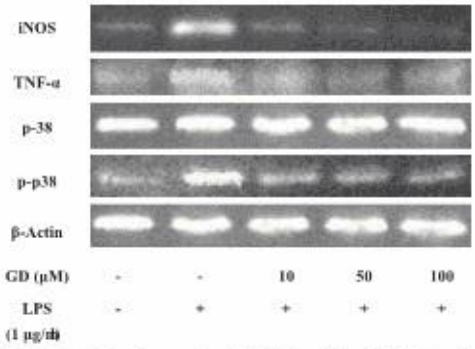


Fig. 6. Effect of GD on the expression of iNOS, TNF-α, p-38, and p-p38 in microglia N9 cells induced by LPS. The iNOS and TNF-α proteins were examined by western blot analysis. Cells were pre-treated with GD (10, 50, and 100 μM) for 20 h and exposed to LPS (1 μg/ml) for 4 h. The data shown represent three independent experiments with comparable outcomes.

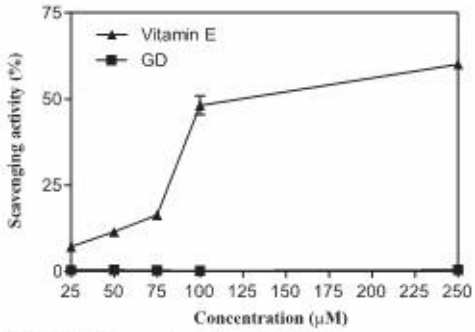


Fig. 7. DPPH radical scavenging activity of GD. GD did not present any scavenger effects on DPPH radicals, suggesting that the antioxidant mechanism of action of GD may not be related to their ability to donate an electron or hydrogen radical. The data shown represent three independent experiments.

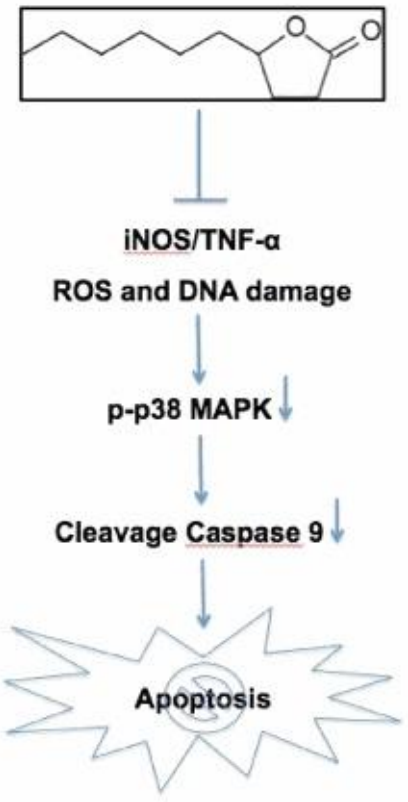


Fig. 8. Schematic representation of the plausible molecular mechanism proposed for the neuroinflammatory protection by GD in N9 cells.

oxygen species production or the production of the expression of iNOS by blocking p38 activation induced by LPS in N9 cells. These effects of GD are significant in the suppression of neurons injury and in the prevention and treatment of several neurodegenerative diseases.

Authors' contributions

PP and CMV carried out all assays in the study, and participated in the drafting of the manuscript. VRC, NAB, and RBS participated evenly in all assays. JS and PP conceived, designed, supervised the study, and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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4. ARTIGO CIENTÍFICO II

Neuroprotective effect of gamma-decanolactone on pilocarpine-induced seizures in mice.

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Neuroprotective effect of gamma-decanolactone on pilocarpine-induced seizures in mice

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Abstract

Gamma-decanolactone (GD) is a monoterpene compound that has activity against seizures induced by pentylenetetrazole and open field tasks. Its mechanism of action is likely to be via glutamate antagonism. GD also inhibits intracellular reactive oxygen species (ROS) generation and the lipopolysaccharide-induced expression of iNOS and TNF-α *in vitro*. Considering the neuropharmacological profile of GD studied so far, we investigated the effect of intraperitoneal administration of 100 and 300 mg/kg of GD on pilocarpine (PIL)-induced seizures in mice. Behavioral, biochemical and oxidative stress parameters were evaluated. DNA damage in the cerebral cortex of mice was assessed by the Comet assay. The results showed that the latency to the first clonic seizure was increased in the groups treated with 300 mg/kg GD. Both doses of GD were able to increase superoxide dismutase and catalase activities and to decrease ROS and nitrite production and DNA damage in the cerebral cortex. These findings demonstrate that GD improves behavioral parameters in the PIL model and protects seizure-related oxidative stress and DNA damage in mice.

Keywords: gamma-decanolactone, genotoxicity, oxidative stress, pilocarpine, seizure.

1. Introduction

Epilepsy is a brain function disorder characterized by unpredictable and recurrent seizures. Its prevalence in the general population is approximately 1% (Świąder et al., 2016), reaching at least 70 million people worldwide (Sucher and Carle, 2015). Temporal dysfunction of neurons culminates in clinical manifestations such as epileptic seizures (Kaur et al., 2015).

Approximately 30% of epileptic patients have temporal lobe epilepsy (TLE) represented by the progressive development of complex partial seizures, hippocampal neurodegeneration and co-morbidities such as cognitive and mood impairments (Shetty and Upadhya, 2016).

Therapeutic strategies available to treat epilepsy have only been able to treat the symptoms and have often proven ineffective (Zhang et al., 2015). Although there are more than 20 antiepileptic drugs (AEDs) available, about one-third of patients still presents drug resistance or adverse effects, increasing the chance to become refractory to pharmacological treatment (Verrotti et al., 2016).

Oxidative stress is described as an imbalance between generation and elimination of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Excess of free radicals production is associated with damage caused to cell structures, including lipid peroxidation, DNA damage and enzyme inactivation. The central nervous system (CNS) becomes highly sensitive due to its high oxygen consumption and the low antioxidant defense activity, resulting in pathogenic processes such as epilepsy (Dey et al., 2016).

In the last decade, clinical and experimental evidence has shown that neuroinflammation is related to epilepsy of different etiologies (Dedeurwaerdere et al., 2012; Kaur et al., 2015; Kobow et al., 2012), especially in TLE, where it is observed an increase in the inflammatory responses and oxidative stress (Rocha et al., 2016; Souza-Monteiro et al., 2015).

In the search for new AEDs, many experimental animal models are used. They may help enrich our understanding of the mechanisms involving the relationship between epilepsy and

behavioral anomalies. Systemic administration of pilocarpine (PIL) in rodents reproduces the main features of human TLE (Lopes et al., 2016). PIL is a muscarinic receptor agonist that induces alterations that lead progressively to status epilepticus (SE) and produces a model highly related to TLE (Curia et al., 2008; Villalpando-Vargas and Medina-Ceja, 2015).

Gamma-decanolactone (GD) is a monoterpene compound that has activity against seizures induced by pentylenetetrazole and open field tasks (Coelho de Souza et al., 1997; Pereira et al., 1997; Pflüger et al., 2016). Its mechanism of action is uncertain, but Pereira et al. (1997) showed a dose-dependent inhibition of glutamate binding in the cortex of rats treated with GD. This antagonism can be associated to the anticonvulsant effect observed *in vivo*, since glutamate receptor activation is increased in some epilepsy models. In addition, our recently findings showed that GD attenuated the activation of N9 cells and inhibited intracellular ROS generation and lipopolysaccharide-induced expression of iNOS and TNF-α in the cells. Moreover, GD blocked the p38 phosphorylation and inhibited cleaved caspase-9 and DNA damage (Pflüger et al., 2016). Thus, this study was aimed to evaluate GD anticonvulsant potential using PIL-induced epileptic mice and to measure its effect on oxidative stress, nitric oxide (NO) production and DNA damage in cerebral cortex of mice.

2. Materials and Methods

2.1. Animals

Adult male CF1 mice of 2-3 months of age and weighing 30-40 g were used. Animals were divided into groups according to the experimental model (N = 10). Mice were housed in plastic cages (five per cage), with water and food *ad libitum*, under a 12 h light/dark cycle (lights on at 8:00 AM), and at a constant temperature of 23 ± 2 °C. All experimental procedures were carried out in accordance to the national and international legislation (Guidelines of Brazilian Council of Animal Experimentation – CONCEA – and EU Directive

2010/63/EU for animal experiments), with the approval of the Committee on the Ethical Use of Animals of Federal University of Rio Grande do Sul (authorization number 28513). All protocols were designed aiming to reduce the number of animals used to a minimum, as well as to minimize their suffering.

2.2. Drugs and pharmacological procedures

GD was purchased from Sigma-Aldrich (St. Louis, USA) and solubilized in Tween 80 (5%). PIL was acquired from Sigma-Aldrich and diazepam (DZP) (Compaz®) was purchased from Cristália (Porto Alegre, Brazil). PIL and DZP were solubilized in 0.9% saline solution and administered intraperitoneally (ip) at a volume of 10 mL/kg body weight.

2.3. Pilocarpine-induced seizure test

The anticonvulsant evaluation of GD on PIL-induced seizures in mice was based on Costa et al. (2012), with minor modifications. DZP (2 mg/kg) was used as a positive control. Mice were kept individually in transparent cages (25 cm X 15 cm X 15 cm) for 30 min to acclimatize to their new environment before each experiment. First, animals received an intraperitoneal injection of saline, tween, DZP or GD (100 and 300 mg/kg). Thirty min after treatment, mice received a single dose of PIL (250 mg/kg) and were observed during 60 min for the latency and percentage of occurrence of clonic forelimb seizures longer than 3 sec. Doses of PIL and DZP were chosen according to previous pilot tests performed in our laboratory. All experiments were carried out between 8 a.m. and 4 p.m. in a quiet room with a room temperature of 22 ± 1°C.

2.4. Tissue homogenization

Frozen cortex from each treated mice was homogenized in ice-cold phosphate buffer (KCl 140 mmol/L, phosphate (20 mmol/L, pH 7.4) and centrifuged at 12,000 rpm for 10 min to obtain the supernatant. Protein concentration was measured according to the method of Lowry et al. (1951) using serum bovine albumin as standard.

2.5. Free radical levels

Homogenates were overlaid with 100 μL of 25 μM dichlorofluorescein diacetate (DCFDA) and were placed back in the incubator for an additional 30 min at 37 °C. At the end of the incubation period, plates were removed and fluorescence of the homogenates was measured on a SpectraMax M2e Microplate Reader (MDS Analytical Technologies, Sunnyvale, CA, USA). The excitation/emission wavelengths for DCFDA were 480/520 nm. Values of relative fluorescence (RFU) were expressed as RFU mg^{-1} protein (Mattiello et al., 2015).

2.6. Superoxide dismutase (SOD) activity

SOD activity was evaluated by quantifying the inhibition of superoxide-dependent autoxidation of epinephrine (Misra and Fridovich, 1972). Briefly, 170 μL of a mixture containing 50 mM glycine buffer (pH 10.2) and 10 mM catalase were added to 20 μL of homogenate. After that, 10 μL of epinephrine were added and the absorbance was immediately recorded in the SpectraMax M2e Microplate Reader every 30 sec for 12 min at an absorbance of 480 nm. The inhibition of epinephrine autoxidation occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically. One SOD unit is defined as the amount of SOD necessary to inhibit 50% of epinephrine autoxidation, and the specific activity is reported as SOD units/mg protein.

2.7. Catalase (CAT) activity

CAT activity was assayed according to the method described by Chance and Machley et al. (1954), based on the disappearance of H_2O_2 at 240 nm. Briefly, 180 µL of 20 mM potassium phosphate buffer (pH 7.2) were added to 10 µL of homogenate. Subsequently, 10 µL of 5 mM H_2O_2 were added and the absorbance was immediately recorded in the SpectraMax M2e Microplate Reader every 30 sec for 10 min. One CAT unit is defined as one µmol of H_2O_2 consumed per minute and the specific activity is calculated as CAT units/mg protein.

2.8. Alkaline comet assay

The alkaline comet assay was performed as previously described by Singh et al. (1988). Briefly, 10 µL of cortex cell suspensions were mixed with 90 µL LMP agarose, spread on a normal agarose precoated microscope slide and placed at 4 °C for 5 min to allow for solidification. Cells were lysed in a high-concentration salt and detergent solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris with 1% Triton X-100 and 10% DMSO freshly added) for 2 h. Slides were removed from lysing solution and washed three times with PBS. Subsequently, cells were exposed to alkali conditions (300 mM NaOH / 1 mM Na_2EDTA, pH > 13, 30 min, 4 °C) to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 25 min at 25 V and 300 mA (94 V/cm). Then, slides were neutralized and silver stained (Nadin et al., 2001). One hundred cells were scored visually according to tail length and the amount of DNA present in the tail. Each comet was given an arbitrary value of 0–4 (0, undamaged; 4, maximally damaged), as described by Collins et al. (1995). Damage score was thus assigned to each sample, ranging from 0 (completely undamaged: 100 cells x 0) to 400 (with maximum damage: 100 cells x 4). International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method, as it is highly correlated with computer-based image analysis (Burlinson et al., 2007; Collins et al., 1995).

2.9. Measurement of NO production

The production of NO was estimated by measuring the amount of nitrite, a stable metabolite of NO (Freitas et al., 2005). Briefly, 100 µL of homogenate was mixed with 100 µL of the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine and 2.5% H_3PO_4) at ambient temperature. After 20 min, absorbance was measured at 540 nm in the SpectraMax M2e Microplate Reader. A nitrite calibration curve was used to convert absorbance to µM nitrite.

2.10. Statistical analysis

The Fisher Exact Test was applied to analyze the percentages of seizures. The latency data were analyzed by the Kruskal-Wallis followed by Dunns ($\alpha = 0.05$). Experiments with homogenates were independently repeated at least three times, with triplicate samples for each treatment. Results were expressed as means ± standard deviations (SD) and submitted to one-way ANOVA and Tukey post test ($\alpha = 0.05$). Statistical analysis was performed using GraphPad Prism 5.0 (San Diego, CA, USA).

3. Results

3.1. Effect of GD on PIL-induced seizures

As shown in Fig. 1A, DZP, used as a positive control, was able to prolong the latency to first clonic seizure induced by PIL ($p \leq 0.001$). Moreover, DZP completely abolished the occurrence of clonic seizures produced by PIL in this study ($p \leq 0.001$). The group receiving 100 mg/kg GD did not present significant difference in all parameters measured compared to the control group. The latency to the first seizure was increased in the group treated with 300 mg/kg GD ($p \leq 0.01$; Fig. 1B). The percentage of seizures was lower in the group that

received 300 mg/kg GD compared to the control group, but this difference was not statistically significant ($p = 0.086$).

3.2. Effect of GD on ROS formation

There is evidence that greater formation of free radicals can occur in the PIL-induced seizure model. Therefore, we assessed intracellular ROS formation in this model of seizures by monitoring the changes in DCFH fluorescence intensity. The results indicated the suppressive effect of GD on DCFH fluorescence production in both tested doses (100 and 300 mg/kg) showing a significant difference from the control group ($p \leq 0.001$; Fig. 2).

3.3. Effect of GD on SOD and CAT activities

In order to investigate whether the antioxidant properties of GD were mediated by an increase in antioxidant enzymes, SOD and CAT activities were measured (Fig. 3A and B, respectively). Mice treated with both doses of GD (100 and 300 mg/kg) showed increased SOD and CAT activities ($p \leq 0.01$ and $p \leq 0.001$, respectively) during oxidative stress promoted by PIL in cerebral cortex, when compared to the control group.

3.4. Effect of GD on DNA-damage

The alkaline comet assay detects DNA strand breaks and alkali-labile sites. Our results showed that PIL-induced seizure promoted significant DNA damage measured in cortex tissue (Fig. 4). GD at both doses (100 and 300 mg/kg) attenuated the generation of this damage compared to the control group ($p \leq 0.05$ and $p \leq 0.001$, respectively).

3.5. Effect of GD on NO production

NO is produced from the amino acid L-arginine by the enzymatic action of nitric oxide synthase (NOS). PIL-induced seizure increased the amount of NO in the cerebral cortex and both doses of GD (100 and 300 mg/kg) were able to inhibit its production ($p < 0.05$ and $p < 0.01$; Fig. 5).

4. Discussion

Oxidative stress, neuroinflammation and epilepsy are thought to be closely interrelated (De Vries et al., 2016, Swiader et al., 2016). Therefore, this study was performed to determine the effect of GD on ROS production, SOD and CAT activities, DNA damage and nitrite content in adult mice after PIL-induced seizures. The results demonstrated that GD exhibited neuroprotective effect in the behavioral model used. This conclusion derives from the following: 1) GD at 300 mg/kg increased the latency for the onset of the first seizure episode when compared to the PIL-treated group; 2) GD protected against the PIL-induced increase in ROS levels; 3) GD protected against PIL-induced inhibition of SOD and CAT activities; 4) GD decreased DNA damage; and 5) GD decreased the release of NO.

PIL-induced seizure, originally described by Turski et al. (1987), is a rodent model of SE that represents an important tool to evaluate behavioral and neurochemical characteristics associated with seizure activity (Clasadonte et al., 2016; Curia et al., 2008; Otsuka et al., 2016). Systemic administration of PIL has been used as an animal model for TLE and has many features in common with human complex partial seizures (Curia et al., 2008).

Our results showed that the group treated with GD at 300 mg/kg had significant increase in the installation time for the first seizure, after the PIL administration. The percentage of seizures was lower in the group that received 300 mg/kg GD compared to the control group, although this result was not statistically significant. These results are similar to other anticonvulsant agents. A previous study showed that sodium valproate (300 mg/kg) increased

the latency to seizure initiation but was ineffective in preventing SE (Curia et al., 2008). Lacosamide, a novel anticonvulsant drug used for the treatment of SE in humans, when tested at a dose of 40 mg/kg, also significantly prolonged latency time to SE in animal models of seizures without affecting the occurrence of seizures (Rapacz et al., 2016).

Epilepsy arises from imbalances between excitatory and inhibitory synaptic transmission, in which fast excitatory neurotransmission is mediated via glutamate receptors (Aseervatham et al., 2016; Smolders et al., 2002). Glutamate is the major excitatory neurotransmitter in the CNS that stimulates both ionotropic and metabotropic receptors. Studies of microdialysis revealed that PIL induces an elevation in glutamate levels in the hippocampus following the appearance of seizures (Smolders et al., 1997). Evidences support the finding that, following initiation by M1 receptors, seizures are maintained by NMDA receptors activation (Curia et al., 2008). Intrahippocampal administration of MK-801, a selective non-competitive NMDA-receptor antagonist, was able to protect against PIL-induced seizures, providing evidence for the involvement of glutamate system in the generation of epilepsy by cholinomimetics (Smolders et al., 1997). Pereira et al. (1997) demonstrated that GD acts as a non competitive glutamate antagonist, and therefore this mechanism could support its effect on latency to first seizure induced by PIL. Moreover, PIL caused depletion of SOD and CAT activities, increased ROS and NO production and promoted DNA damage, suggesting imbalance between oxidants and weakening of oxidative defense and, consequently, oxidative damage. As mentioned above, during SE there is free radicals over-formation and⁄or a deficiency of antioxidant systems, as evidenced by the augmented nitrite content, all of which characterize oxidative stress.

Our findings corroborate previous studies in our laboratory demonstrating that GD inhibits iNOS and TNF-alpha production by lipopolysaccharide-activated microglia in N9 cells (Pflüger et al., 2016). GD also showed a dose-dependent suppression of ROS production

without presenting any scavenger effect on DPPH radicals, suggesting that the antioxidant mechanism of GD action may not be related to its ability to donate an electron or hydrogen radical. Probably, this suppression is related to the p38 MAPK signaling pathway (Pflüger et al., 2016).

In normal conditions, there is a steady-state balance between the production of NO and metabolites (nitrite and nitrate) and their destruction by antioxidant systems. Other studies have shown that nitrite and nitrate concentrations are not elevated in epileptic patients (Vanhatalo and Riikonen, 2001). Other mechanisms may be associated with the increase in ROS levels as well as in neurodegeneration observed in epileptic humans (Mello Filho et al., 1984; Pazdernik et al., 2001). Our results show an increase in nitrite formation after SE, suggesting that there is a possible increase in ROS concentrations, which are often involved in neuronal damage. PIL was able to promote DNA damage, most likely by increasing the release of glutamate, leading to excitotoxicity and oxidative stress in brain tissues (Yang et al. 2011). Treatment with both doses of GD induced a protective effect against DNA damage in cortex. In this model, DZP, which is an important drug used in epilepsy treatment, was also able to prevent genotoxic damage. DNA is also a major target of constant oxidative damage from endogenous and exogenous oxidants (Ercegovac, 2010). Therefore, the results obtained here suggest that GD could produce neuronal damage protection, contributing to decrease injury in cerebral cortex associated to epilepsy.

In summary, this study demonstrated that GD was able to protect against PIL-induced seizures, oxidative stress and DNA damage in mice. The results suggest that GD has potential as anticonvulsant drug and deserves to be investigated in other seizure models as well as in different processes associated to epilepsy.

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Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1. Gamma-decanolactone (GD) effect (100 and 300mg/kg) on PIL-induced seizures in mice. **(A)** Latency to first clonic seizure; **(B)** percentage of clonic seizure (n = 10). *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ versus saline (Sal) group. Latency data were analyzed by Kruskal-Wallis/Dunns and seizures by Fisher's exact test.

Figure 2. Gamma-decanolactone (GD) effect on PIL-induced oxidative stress in mice cerebral cortex. More oxidizing extracellular conditions demonstrated an increase in DCF fluorescence and are indicative of an increase in intracellular ROS generation. Values represent the mean ± SD of six animals per group. ***$p \leq 0.001$ compared to the PIL group; [a]compared to Sal/Sal group.

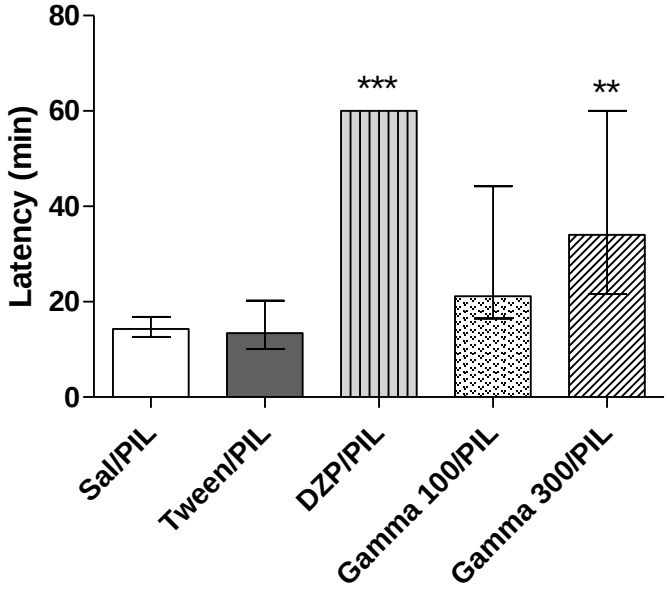
Figure 3. Gamma-decanolactone (GD) effect on SOD (**A**) and CAT (**B**) enzyme activities in mice cerebral cortex. Values represent the mean ± SD of six animals per group. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared to the PIL group; [a]compared to Sal/Sal group.

Figure 4. Gamma-decanolactone (GD) effect on PIL-induced DNA damage in mice cerebral cortex. Values represent the mean ± SD of six animals per group. *$p \leq 0.05$, ***$p \leq 0.001$ compared to the PIL group; [a]compared to Sal/Sal group.

Figure 5. Gamma-decanolactone (GD) effect on PIL-induced NO production in cerebral cortex of mice. Values represent the mean ± SD of three animals per group. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared to the PIL group; [a]compared to Sal/Sal group.

Figure 1

A


80
60
40
20
0
Latency (min)
***
**
Sal/PIL
Tween/PIL
DZP/PIL
Gamma 100/PIL
Gamma 300/PIL

B

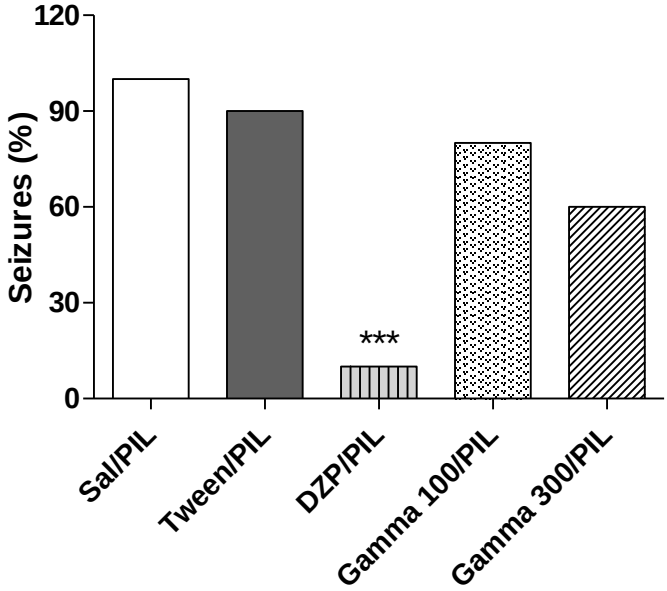

120
90
60
30
0
Seizures (%)
***
Sal/PIL
Tween/PIL
DZP/PIL
Gamma 100/PIL
Gamma 300/PIL

Figure 2

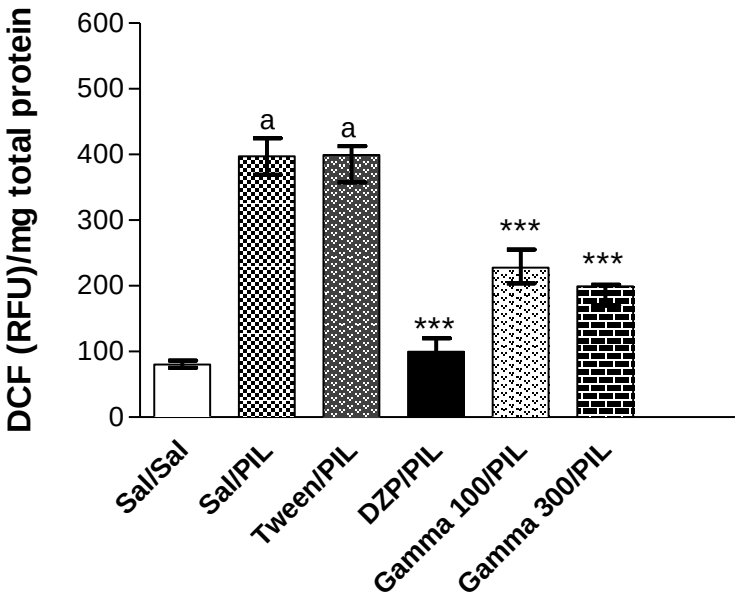
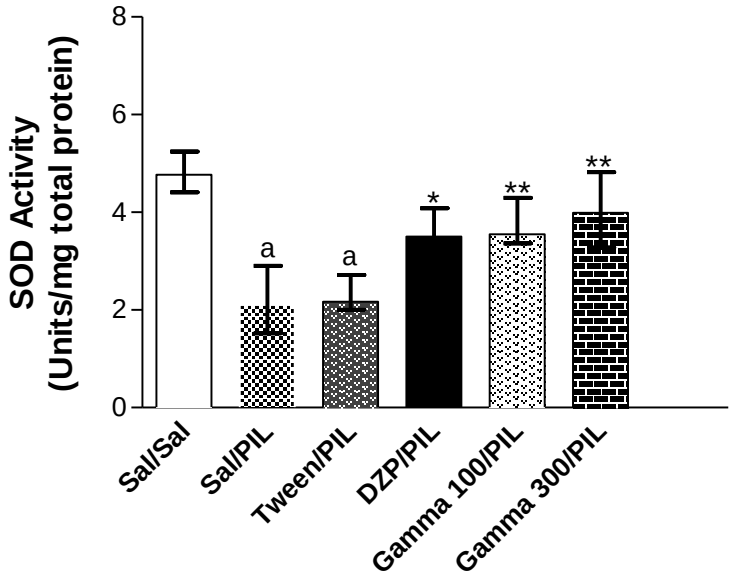

DCF (RFU)/mg total protein
600
500
400
300
200
100
0
a
a
***
***
***
Sal/Sal
Sal/PIL
Tween/PIL
DZP/PIL
Gamma 100/PIL
Gamma 300/PIL

Figure 3

A


SOD Activity
(Units/mg total protein)
8
6
4
2
0
a
a
*
**
**
Sal/Sal
Sal/PIL
Tween/PIL
DZP/PIL
Gamma 100/PIL
Gamma 300/PIL

B

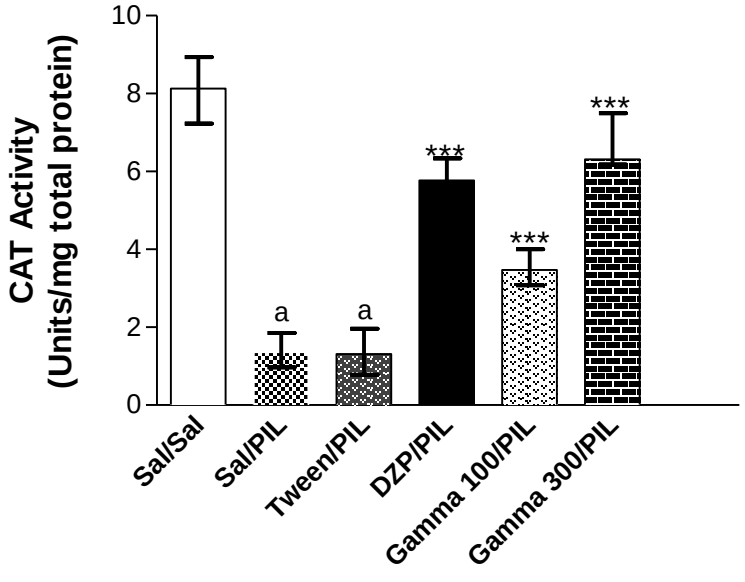

CAT Activity
(Units/mg total protein)
10
8
6
4
2
0
a
a
***
***
***
Sal/Sal
Sal/PIL
Tween/PIL
DZP/PIL
Gamma 100/PIL
Gamma 300/PIL

Figure 4

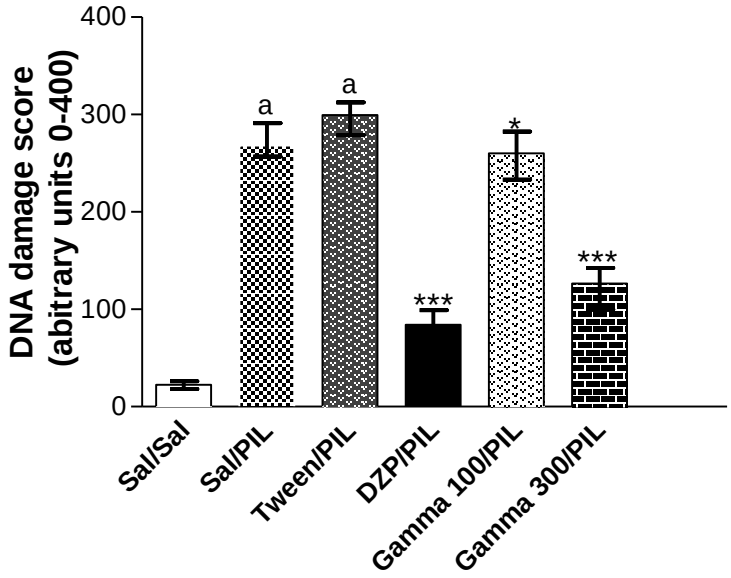
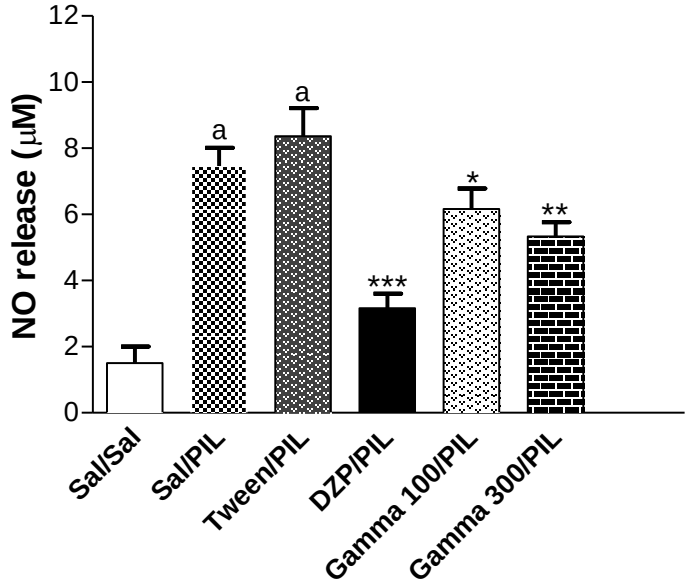


Figure 5



5. ARTIGO CIENTÍFICO III

Neuropharmacological evaluation of gamma-decanolactone in different epilepsy mouse models.

Será submentido a revista: Epilepsy & Behavior

Neuropharmacological evaluation of gamma-decanolactone in different epilepsy mouse models

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Abstract

Gamma-decanolactone (GD) is a monoterpene compound that presents anticonvulsant effect in acute and chronic models of epilepsy (PTZ-induced) and acts as a noncompetitive glutamate antagonist in binding assays. This study aimed to explore the anticonvulsant profile of GD in seizures induced by aminophylline (AMPH; 280 mg/kg), isoniazid (INH; 250 mg/kg), picrotoxin (PCT; 5 mg/kg) and 4-aminopyridine (4-AP; 13 mg/kg) in male mice. Thirty minutes before the administration of the convulsant agents, animals received a single administration of saline, GD (100 or 300 mg/kg) or the positive control diazepam (2 mg/kg). The parameters evaluated during 1 h were the latency to first seizure, occurrence of seizure and mortality rate. The rotarod performance test was used to evaluate the neurotoxicity of GD at a dose of 300 mg/kg. GD was also evaluated in the DZP-induced sleep test. DZP increased the latency to first seizure in all seizure tests and decreased the percentage of seizures in three of the four behavioral models. The results demonstrated that GD was able to prolong the latency to first seizure induced by AMPH, INH and 4-AP, but not by PCT. GD administration was able to reduce the latency to fall off the rotarod test only at the time of 30 min. In the DZP-induced sleep test, GD shortened the onset and prolonged the time of sleep. The results indicate that GD: (1) presents anticonvulsant effects in different epilepsy mouse models; (2) GD could modulate adenosine and GABA pathways and affect potassium channels directly or indirectly.

Key words: aminophylline, 4- aminopyridine, gamma-decanolactone, isoniazid, picrotoxin, seizure.

Introduction

Epilepsy is the most common chronic neurological disorder, affecting approximately 70 million people worldwide [1-3]. Approximately 30% of epileptic patients have temporal lobe epilepsy (TLE), represented by progressive development of complex partial seizures, hippocampal neurodegeneration and co-morbidities such as cognitive and mood impairments [4]. Current treatments have been often unsuccessful in blocking seizures. Unfortunately, not all patients respond well to pharmacological treatments available, as well as suffer from the adverse effects caused by anticonvulsant drugs [5, 6]. About 30–40% of patients typically develop pharmacoresistant or intractable epilepsy [4]. Thus, the development of more effective and safe pharmacological approaches remains an important issue.

Natural products represent an important source of molecules with biological activity. Many plants are used in traditional treatments of different diseases, such as epilepsy [7]. Moreover, synthetic compounds are tested for their structural similarities with bioactive components of plant species.

The search of new molecules with biological properties, such as anticonvulsant activity, includes the group of the lactones. Gamma-decanolactone (GD) is a monoterpene compound, structurally similar to lactones found in the essential oil of *Aeollanthus suaveolens*, a species commonly used by the Amazonian population as medicine [5]. GD showed to be active in some behavioral models, such as acute pentylenetetrazole (PTZ)-induced seizures, PTZ-kindling and open field test [5, 9, 10]. Moreover, using binding studies in mouse cortex homogenate, it was demonstrated that GD acts as a non-competitive antagonist of glutamate, an important mechanism of action considering the involvement of glutamatergic system in epilepsy [10]. Recently, we demonstrated that GD attenuated the activation of N9 cells and inhibited intracellular reactive oxygen species and the expression of iNOS and TNF-α induced by lipopolysaccharide (LPS). Also, GD blocked the phosphorylation of p38 and inhibited

cleaved caspase-9 and DNA-damage [11]. Thus, the goal of this study was to evaluate GD anticonvulsant effect in epilepsy models induced by different agents: aminophylline (AMPH), isoniazid (INH), picrotoxin (PCT) and aminopyridine (4-AP). Additionally, GD was evaluated in the rotarod and diazepam(DZP)-induced sleep time tests.

Materials and methods

Animals

We used in this study 208 CF1 male mice (2–3 months of age, 30–40 g) obtained from the biotery of the Federal University of Rio Grande do Sul (UFRGS). The animals were divided into groups according to the experimental model (n = 7–10). Mice were housed in plastic cages (five per cage), with water and food ad libitum, under a 12-h light/dark cycle (lights on at 8 a.m.), and at a constant temperature of 23 ± 2 °C. All experimental procedures were carried out in accordance with the national and international legislation (guidelines of Brazilian Council of Animal Experimentation – CONCEA – and of EU Directive 2010/63/EU for animal experiments), with the approval of the Committee on the Ethical Use of Animals of the UFRGS (authorization number 28513). All protocols were designed aiming to reduce the number of animals used to a minimum, as well as to minimize their suffering.

Drugs and pharmacological procedures

GD was purchased from Aldrich Chemical Co. and was solubilized in 5% Tween 80. AMPH, INH, PCT and 4-AP were acquired from Sigma-Aldrich (St. Louis, USA), and DZP (Compaz®) was purchased from Cristália (Brazil). All the drugs were solubilized in 0.9% saline and administered intraperitoneally (ip) at a volume of 10 ml/kg body weight.

Anticonvulsant activity assessment

To evaluate the anticonvulsant effect of GD in mice we used four different convulsant agents: AMPH (280 mg/kg), INH (250 mg/kg), PCT (5 mg/kg) and 4-AP (13 mg/kg), according to previous studies [6, 12-14]. DZP (2 mg/kg) was used as positive control in all seizure tests. Briefly, mice were kept individually in transparent mice cages (25 cm × 15 cm × 15 cm) for 30 min to acclimatize to their new environment before the commencement of each experiment. Initially, animals received an ip injection of saline, Tween 80, DZP or GD (100 or 300 mg/kg). After 30 min, a second injection of AMPH, INH, PCT or 4-AP was administered. The animals were observed for seizures occurrence for periods of 1 h post-treatment with AMPH, INH or 4-AP, and 30 min post-treatment with PCT. The parameters observed were the occurrence of clonic/tonic-clonic seizures, and the latency of onset of convulsions (min). Mortality was evaluated at 1 h after the AMPH, INH and 4-AP administration. All experiments were carried out between 8 a.m. and 4 p.m. in a quiet room with an ambient temperature of 22 ± 1 °C.

Rotarod test

The method described by Leal et al. [15], with minor modifications, was used in this study to assess motor coordination in mice treated with GD. Mice were initially trained to remain on the rotarod apparatus (Ugo Basile Rota-Rod, Model 7650, Jones and Roberts, Italy) at 18 rpm for 120 sec; those that did not remain on the bar for at least two out of three consecutive trials were discarded. Selected mice were divided into four groups (n=8): group I, saline; group II, 5% Tween 80; group III, 100 mg/kg GD; and group IV, 300 mg/kg GD. The latency to fall from the rotarod (one trial of maximum 60 sec) was determined 30, 60, 90 and 120 min after the administration of the drugs.

DZP-induced sleep test

This test was performed according Chindo et al. [6], with minor modifications. Mice received the following treatments: saline, GD 100 mg/kg e 300 mg/kg. Thirty minutes after treatments, DZP (17 mg/kg) was administered to all groups. Each mouse was observed for the onset (onset to sleep) and duration of sleep (time of sleep). The criterion for sleep was defined as the loss of righting reflex [16], indicated by the animal inability to return to its upright position on all four limbs after being gently rolled sideways.

Statistical analysis

The results of percentage of seizures and mortality were analyzed by Fisher exact test. The latency data were analyzed by Kruskal–Wallis followed by Dunn's test. A value of $p < 0.05$ was considered significant. Results of the rotarod test were analyzed by ANOVA followed by Duncan´s test. Results of the rotarod test were expressed as mean ± SEM. The results of DZP-induced sleep test were analyzed by Kruskal–Wallis one-way ANOVA followed by Dunn's test.

Results

As shown in Fig. 1, DZP (2 mg/kg), used as positive control, was able to prolong the latency to first tonic-clonic seizures induced by AMPH ($p \leq 0.001$). Indeed, DZP completely abolished the tonic-clonic seizures occurrence and the mortality parameter caused by AMPH administration ($p \leq 0.001$).

The latency to the first seizure was increased in the groups treated with 100 or 300 mg/kg GD ($p \leq 0.05$). GD, in none of the doses tested, was able to reduce the percentage of seizures or mortality when compared to the control, although both GD groups have shown lower values ($p = 0.0698$).

The effect of GD in the INH-induced seizures in mice is shown in Fig. 2, DZP, a $GABA_A$-BZD antagonist, was able to increase the latency to the first seizure ($p \leq 0.05$), but did not change other parameters (percentage of seizures or mortality). The group receiving 100 mg/kg GD did not show significant differences in any parameters when compared to the control group. The animals treated with 300 mg/kg GD had a significant increase in latency ($p \leq 0.001$) and a reduction in mortality ($p \leq 0.05$). In the percentage of seizures, GD at 300 mg/kg presented a lower mean compared to the control group, but this difference was not statistically significant ($p = 0.0769$).

As observed in Fig. 3, DZP was able to significantly increase the latency to the first seizure induced by PIC and to reduce the occurrence of seizures ($p \leq 0.001$). Groups treated with GD showed no difference from the control group in both parameters evaluated in this model.

In the evaluation of seizures induced by 4-AP (Fig. 4), DZP prolonged the latency to first seizure ($p \leq 0.01$) and reduced the occurrence of clonic seizures ($p \leq 0.05$). DZP was also able to significantly decrease mortality ($p \leq 0.05$). GD at 300 mg/kg was able to decrease the clonic seizure occurrence ($p \leq 0.05$) and to prolong the latency to first seizure ($p \leq 0.05$) when compared to the control group. Although, GD has not significantly reduced the mortality of the animals, we can clearly observe that the mortality rate was lower in the groups that received 100 or 300 mg/kg GD in comparison to the saline group ($p = 0.0698$).

We can observe in the Fig. 5 that 300 mg/kg GD produced motor impairment up to 30 min after administration ($p < 0.01$), but not thereafter (60, 90 or 120 min) in the rotarod test.

The results obtained in the DZP-induced sleep test (Fig. 6) show that animals injected with saline and DZP (17 mg/kg) showed loss of the straightening reflex within 5 to 10 min of administration. GD at 300 mg/kg but not at 100 mg/kg significantly shortened the onset and prolonged the time of sleep in mice ($p < 0.05$).

Discussion

This study was performed to assess the GD anticonvulsant profile. To address this issue, we used four models of seizures induced by distinct convulsant agents. These chemoconvulsants are widely used to induce convulsions in animal models, which serve as a powerful tool for the development of potential anticonvulsant drugs and to explore the underlying mechanism(s) for their actions [17].

Adenosine is an important endogenous modulator of neuronal excitability that provides anticonvulsant effects. One emerging strategy in the search for new antiepileptic drugs has been to target the adenosine pathway [14, 18]. On the other hand, several studies have shown that xanthines present convulsant activity. This effect seems to be related to their ability in producing stimulation of the central nervous system (CNS) through non-selective antagonism of adenosine receptors, alteration of intracellular calcium concentration and inhibition of cerebral 5'-nucleotidase activity leading to decreased adenosine content [12].

The results revealed that GD prolonged the latency in the AMPH-induced seizure, but it did not significantly reduce the percentage of seizures or mortality rate, although it is possible to observe a decrease in these parameters compared to the control group. This result could suggest that GD is an adenosine receptor agonist and that this could underlie the effect observed in this seizure model. Agonists of A1 and A2 adenosine receptors could potentially be therapeutic agents for a variety of diseases, including epilepsy, since this pathway is implicated in the pathogenesis of this disease. The action of GD on this neurotransmitter system needs further investigation.

Isoniazid induces seizures by interfering with GABA synthesis through inhibition of glutamic acid decarboxylase (GAD) activity, which leads to a rapid depletion in GABA content [6, 13]. GD did not protect the mice against INH-induced seizures, but GD at 300 mg/kg significantly prolonged the latency to the first seizure and markedly reduced the mortality rate. These

results suggest that GD could improve GAD activity, resulting in an increase of the inhibitory GABA-mediated transmission. GABA is the most important inhibitory neurotransmitter of mammal's CNS while glutamate is a major excitatory pathway in the brain. The imbalance between excitatory and inhibitory systems is directly related to seizure occurrence [14]. Previous studies have shown that acute or chronic administration of GD is able to block PTZ-induced convulsions [8, 9]. Moreover, GD showed to be a non competitive antagonist of glutamate receptors [10]. PTZ is able to induce seizures by blocking the major inhibitory pathway mediated by GABA transmitter. Furthermore, several biochemical and behavioral studies suggest the involvement of glutamatergic mechanisms in PTZ-induced seizures [6].

PCT, a $GABA_A$ receptor antagonist, can lead to seizures by blocking the chloride ion channel linked to $GABA_A$-receptors, which is known to be resistant to most anticonvulsant agents [13, 17]. GD at both tested doses failed to protect the animals against PCT-induced seizures, suggesting that it is not able to modulate the specific PCT site in the $GABA_A$-receptor to reopen the closed chloride ion gates.

4-AP is a potent inhibitor of voltage-gated potassium channels causing the release of neurotransmitters in the brain that ultimately leads to convulsions [2]. The present study demonstrated that GD, at the highest dose tested, was able to prolong the latency to the first seizure and to reduce the mortality induced by 4-AP. This effect might be due to the direct activation of potassium channels and their conductance. As mentioned before, GD prolonged the latency to seizure in the INH model; therefore, it is possible that this compound allowed GABA content to increase potassium conductance, suggesting in this case an indirect mechanism of GD on these ion channels.

The effect of the highest dose of GD in mice was evaluated in the rotarod test. The results obtained suggest that GD produces a transient pattern of motor deficit (observed only after 30 min of administration), which might be explained by its sedative effect previously reported in

another study [8] and confirmed here by the ability of 300 mg/kg GD to significantly shorten the onset and increase the time of DZP-induced sleep in mice. Many neurotransmitters in the brain, such as the GABA system, play important roles in sleep mechanisms. Activation of the $GABA_A$ receptor channel complex by the major inhibitory neurotransmitter (GABA) in the CNS is known to favor sleep [6]. It is possible that the effect of GD observed here in the DZP-induced sleep test can be related to its ability to modulate GABAergic system.

It is possible to consider that the anticonvulsant activity of a novel compound is not measured only by its ability to prevent convulsions but also to delay the onset of seizures or to reduce death rate and/or to decrease the frequency of the episodes [17]. The outcome of this study provides evidence that GD possesses anticonvulsant activity. Among the parameters measured, the latency to seizure onset seemed to be the most sensitive to GD anticonvulsant effects. This can suggest that the most potent effect of GD is to slow or block the synchronization or speed of spreading epileptiform activity. Moreover, it was effective in three of the four screening tests used here, similar to DZP effect, which could implies its pharmaceutical potential for a broad spectrum of seizure types.

In conclusion, the data obtained in this study suggest that GD presented anticonvulsant activity in the AMPH, INH and 4-AP models, which could be related to its ability to modulate adenosine receptors and the activity of GAD enzyme and to activate, either directly or indirectly, potassium channels. Therefore, GD is a promising candidate for further preclinical studies aimed at treating epilepsy. Further studies are ongoing in our laboratory to confirm the mechanism of action of GD as well as its effect on oxidative stress and on cell parameters.

Conflicts of interest

We wish to confirm that there are no conflicts of interest associated with this publication and there has been no significant financial support for this study that could have influenced its outcome.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; N. 307064/2013-1) and Federal University of Rio Grande do Sul (UFRGS, N. 28513)

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Figure legends

Figure 1 Effect of GD (100 and 300 mg/kg) on AMPH-induced seizures in mice. (A) Latency to first tonic-clonic seizure; (B) Percentage of tonic-clonic seizures; (C) Percentage of mortality. N = 7-10 mice/group; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ versus saline (SAL) group. Latency data were analyzed by Kruskal-Wallis/Dunn's and seizure and mortality by Fisher's exact test.

Figure 2**.** Effect of GD (100 and 300 mg/kg) on INH-induced seizures in mice. (A) Latency to first clonic seizure; (B) Percentage of tonic-clonic seizures; (C) Percentage of mortality. N = 7-10 mice/group; *$p \leq 0.05$ and **$p \leq 0.01$ versus SAL group. Latency data were analyzed by Kruskal-Wallis/Dunn's and seizure and mortality by Fisher's exact test.

Figure 3**.** Effect of GD (100 and 300 mg/kg) on PCT-induced seizures in mice. (A) Latency to first clonic seizure; (B) Percentage of tonic-clonic seizures. N = 10 mice/group; $p \leq 0.001$ versus SAL group. Latency data were analyzed by Kruskal-Wallis/Dunn's and seizure by Fisher's exact test.

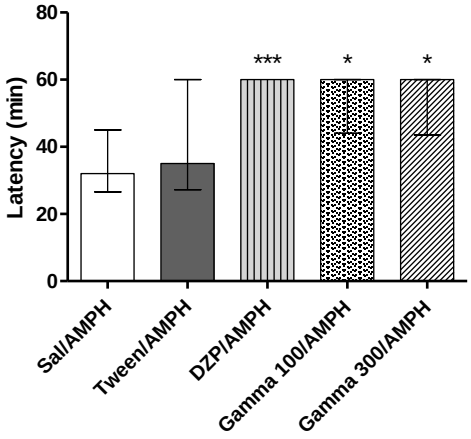
Figure 4. Effect of GD (100 and 300 mg/kg) on 4-AP-induced seizures in mice. (A) Latency to first clonic seizure; (B) Percentage of tonic-clonic seizures; (C) Percentage of mortality. N = 7-10 mice/group; *$p \leq 0.05$ and **$p \leq 0.01$ versus SAL group. Latency data were analyzed by Kruskal-Wallis/Dunn's and seizure and mortality by Fisher's exact test.

Figure 5. Effect of GD (300 mg/kg) on rotarod test at 30, 60, 90 and 120 min post- treatment. Latency is expressed as mean ± SEM (n = 8). **p < 0.01; ANOVA followed by Duncan's test.

Figure 6. Effect of GD (100 and 300 mg/kg) on diazepam-induced sleep in mice. A) onset of sleep, B) time of sleep. N = 10 mice/group; *p ≤ 0.05 versus SAL group. Data were analyzed by Kruskal-Wallis/Dunn’s.

Figure 1

A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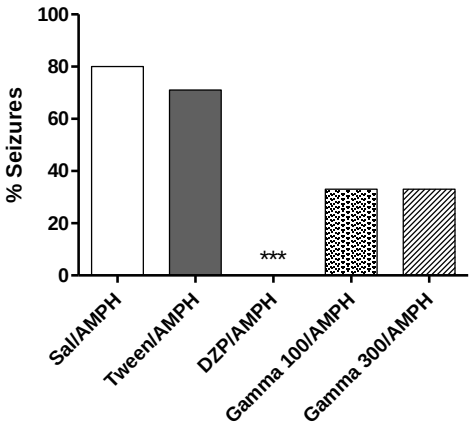
Gamma 300/AMPH

B


% Seizures
100
80
60
40
20
0
***
Sal/AMPH
Tween/AMPH
DZP/AMPH
Gamma 100/AMPH
Gamma 300/AMPH

C

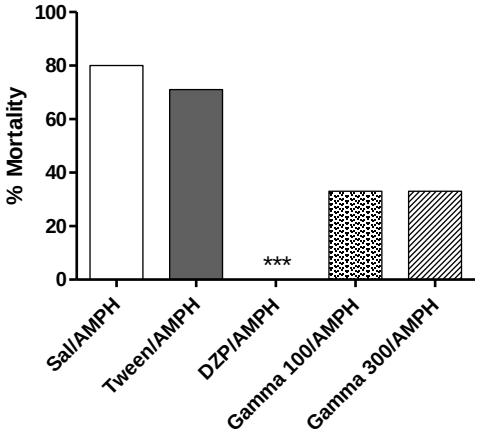
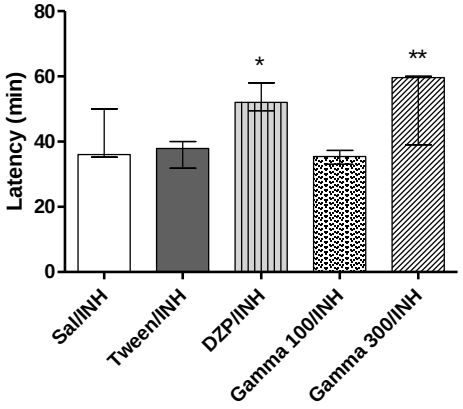
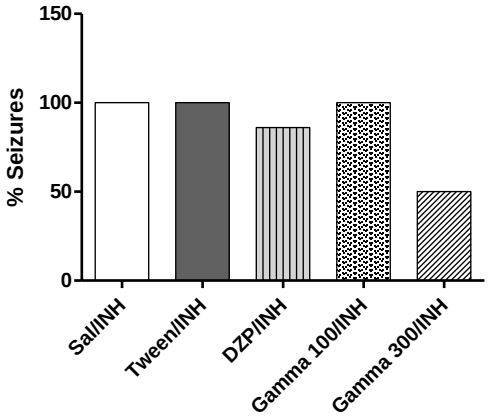

% Mortality
100
80
60
40
20
0
***
Sal/AMPH
Tween/AMPH
DZP/AMPH
Gamma 100/AMPH
Gamma 300/AMPH

Figure 2

A


Latency (min)
80
60
40
20
0
*
**
Sal/INH
Tween/INH
DZP/INH
Gamma 100/INH
Gamma 300/INH

B


% Seizures
150
100
50
0
Sal/INH
Tween/INH
DZP/INH
Gamma 100/INH
Gamma 300/INH

C

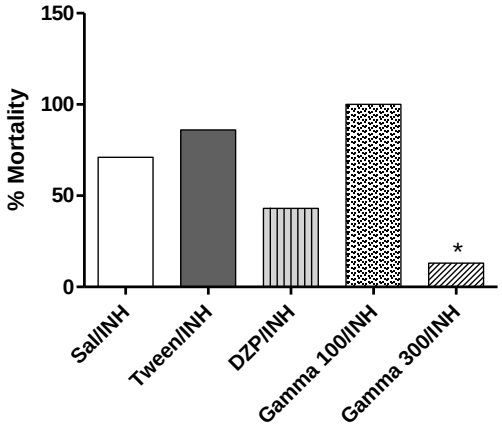
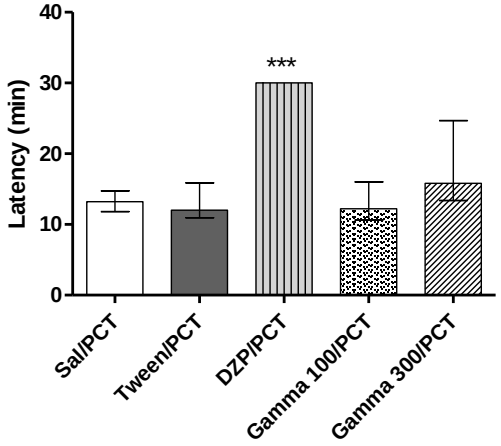

% Mortality
150
100
50
0
*
Sal/INH
Tween/INH
DZP/INH
Gamma 100/INH
Gamma 300/INH

Figure 3

A


Latency (min)
40
30
20
10
0
***
Sal/PCT
Tween/PCT
DZP/PCT
Gamma 100/PCT
Gamma 300/PCT

B

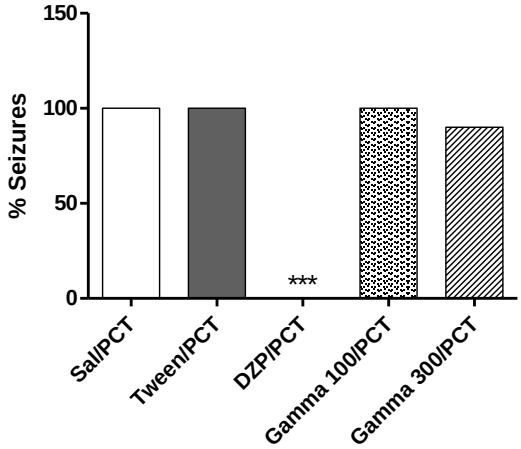
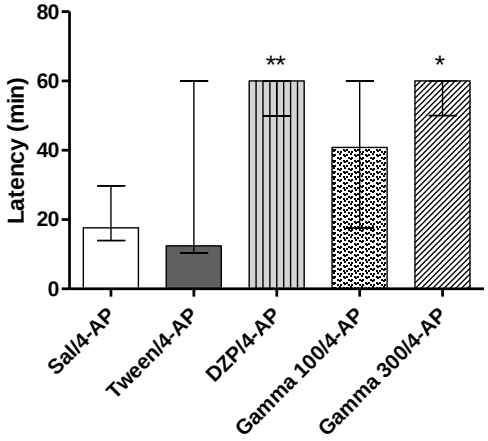
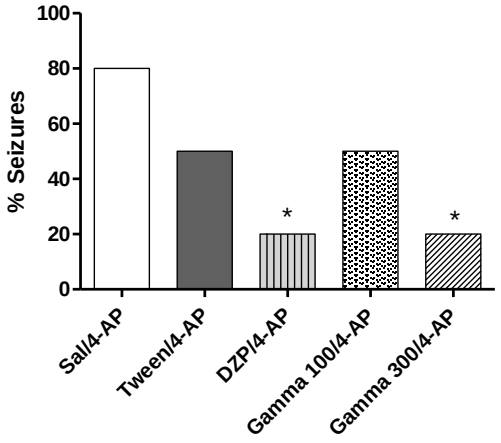

% Seizures
150
100
50
0
***
Sal/PCT
Tween/PCT
DZP/PCT
Gamma 100/PCT
Gamma 300/PCT

Figure 4

A


Latency (min)
80
60
40
20
0
**
*
Sal/4-AP
Tween/4-AP
DZP/4-AP
Gamma 100/4-AP
Gamma 300/4-AP

B


% Seizures
100
80
60
40
20
0
*
*
Sal/4-AP
Tween/4-AP
DZP/4-AP
Gamma 100/4-AP
Gamma 300/4-AP

C

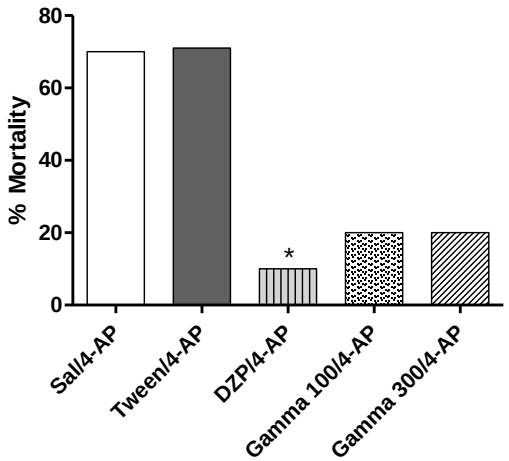

% Mortality
80
60
40
20
0
*
Sal/4-AP
Tween/4-AP
DZP/4-AP
Gamma 100/4-AP
Gamma 300/4-AP

Figure 5

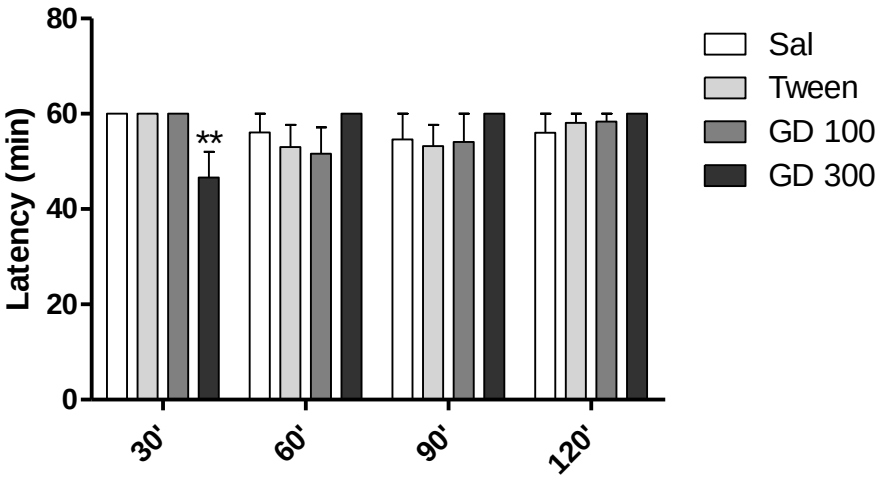
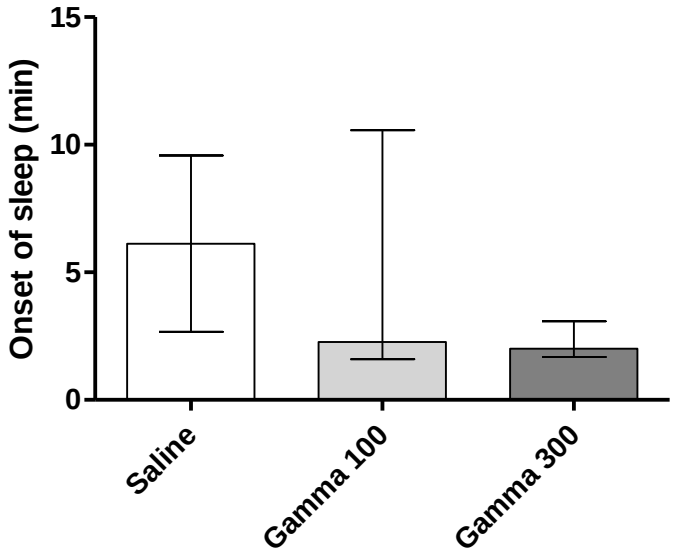
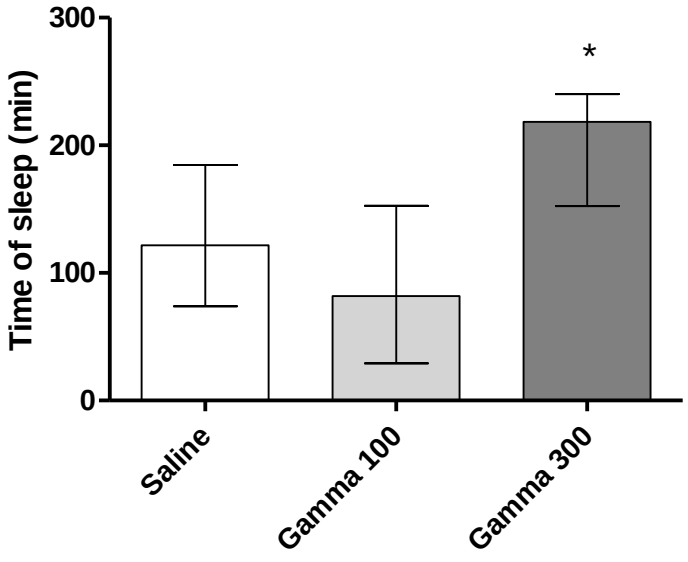


Figure 6

A


Onset of sleep (min)
15
10
5
0
Saline
Gamma 100
Gamma 300

B


Time of sleep (min)
300
200
100
0
*
Saline
Gamma 100
Gamma 300

6 DISCUSSÃO

Epilepsia é uma desordem crônica caracterizada por convulsões recorrentes, a qual, a fisiopatologia ainda não é completamente compreendida, tornando-se uma patologia multifatorial. Evidências sugerem uma relação intrínseca entre epilepsia, neuroinflamação, estresse oxidativo, excitotoxicidade e morte neuronal (VRIES *et al*, 2016, SWIADER *et al*., 2016).

Apesar da falta de evidências se o estresse oxidativo e a neuroinflamação são causas ou consequências da epilepsia, sabe-se que um excesso na produção de radicais livres e a liberação de citocinas pró-inflamatórias mediadas por células gliais, podem levar a convulsões prolongadas. Esse processo resultaria numa disfunção mitocondrial em tecidos cerebrais que procedem à morte neuronal e subsequente epileptogênese (DEVINSKY *et al*., 2013, CARMONA-APARICIO *et al*., 2015).

Sabe-se que as células microgliais desempenham um papel fundamental tanto em processos fisiológicos, quanto em fisiopatológicos do SNC. Especialmente na epilepsia, sabemos que após crises convulsivas, ocorre ativação microglial e aumento de marcadores como, ionized calcium binding adaptor molecule (Iba-1), CD68 uma glicoproteína expressa em monócitos/macrófagos, utilizado como marcador microglial M1, CD11b marcador expresso também em leucócitos, além de receptores purinérgicos, citocinas pró-inflamatórias como L-1b, IL-6, TNF-α e enzimas como COX e iNOS (EYO *et al*., 2016; DEY *et al*., 2016).

Os achados desse estudo estão apresentados em três artigos, inicialmente foi realizado um estudo *in vitro* com células microgliais N9 no modelo clássico de LPS, a fim de mimetizar o efeito neuroinflamatório causado pela epilepsia. Primeiramente, avaliou-se a citotoxicidade de diferentes concentrações de GD em células mirogliais N9 e verificou-se que a GD não apresenta nenhum comprometimento na viabilidade e integridade celular, mostrado com base no ensaio de azul de tripan.

Após, testou-se o efeito da GD sobre a produção de EROS induzidas por LPS e verificou-se que nas doses de 50 e 100μM houve uma diminuição na formação de EROS. Para um melhor entendimento dessa atividade, foi realizado o ensaio de DPPH e verificou-se que a atividade antioxidante de GD não é através da captura de

elétrons livres, uma vez que a sua estrutura molecular não tem capacidade de doar elétrons.

Para investigar se a GD neuromodula a inflamação, foi estudado o seu efeito sobre a inibição de citocinas pró-inflamatórias, na qual novamente nas doses de 50 e 100µM, a GD inibiu a formação de TNF-α, iNOS, caspase 9 clivada e reduziu o dano ao DNA. Sabe-se que a presença de TNF-α na microglia, evoca liberação do glutamato, através do aumento da glutaminase, encarregada de converter glutamina em glutamato (IORE *et al.*, 2016), já a produção de EROS e NO, causam morte neuronal (HU *et al*.,1997) e uma das caspases iniciadoras que possui um papel fundamental na morte celular é a caspase 9 clivada que posteriormente irá ativar as caspases efetoras 3 e 7 resultando no processo apoptótico, essa cascata é regulada por inibidores de caspases (CHUANG *et al*., 2007). Com o intuito de esclarecer a ação de GD sobre a diminuição de EROS, iNOS, TNF-α, dano ao DNA e parâmetros apoptóticos, foi investigado a sua ação sobre o bloqueio da fosforilação de p38 MAPK, uma vez que essa proteína é fortemente ativada por agentes estressores, inflamatórios, genotóxicos e é responsável pela regulação da apoptose (SUI *et al*., 2014).

Tendo em vista que a GD, demonstrou possuir ação no bloqueio da p38 MAPK, acredita-se que esse pode ser um novo alvo para tratamento de desordens neuroinflamatórias. Por fim, conclui-se que os resultados *in vitro*, foram bastante significativos, para um melhor entendimento do possível efeito anticonvulsivante apresentado nos modelos *in vivo*.

No segundo artigo, foi avaliada a atividade anticonvulsivante de GD nas doses de 100 e 300 mg/kg em camundongos, no modelo de pilocarpina e alguns parâmetros bioquímicos ocasionados no córtex.

Sabe-se que o SE induzido pelo modelo de pilocarpina, agonista colinérgico muscarínico está associado com neurodegeneração. Outro dado relevante é que lesões neuronais e neuroinflamação têm sido propostas na patogênese de SE induzidas por pilocarpina (REDDY e KURUBA, 2013). Estudos sugerem que após a administração de pilocarpina em ratos, foi observado um aumento de EROS no hipocampo, caracterizado por uma diminuição no nível das enzimas antioxidantes e aumento no teor de nitritos (DEY *et al*., 2016). Em condições normais, existe um equilíbrio entre a produção de NO e seu metabólitos (nitritos e nitrato) e a sua destruição pelas enzimas antioxidantes (VANHATALO e RIIKONEN, 2001). Nos

nossos achados, constatou-se que após insulto gerado por SE, houve aumento da formação de nitrito, o que pode estar relacionado com o aumento de ROS e dano neuronal.

Wang *et al*., (2016), observaram que as isoformas da enzima GAD (GAD65|GAD67) estão diminuídas em pacientes com ELT e em camundongos submetidos a convulsões induzidas por pilocarpina, uma vez que inibi-se a enzima GAD, reduz-se a síntese do neurotransmissor inibitório GABA. Supostamente se a GD modulasse a enzima GAD, poderia estar aumentado o GABA.

Nos resultados do modelo de pilocarpina, a GD na dose de 300 mg/kg aumentou a latência para primeira convulsão e nas doses de 100 e 300 mg/kg atenuou o desequilíbrio gerado entre radicais livres e enzimas antioxidantes e consequentemente diminuiu o dano ao DNA, quando comparado ao controle positivo DZP. GD não diminuiu a porcentagem de convulsões, assim como outros fármacos anticonvulsivantes clássicos como o valproato de sódio (300 mg/kg) e lacosamida (40 mg/kg) que em outros estudos, também só aumentaram a latência para primeira convulsão, mas não diminuíram a porcentagem de convulsões (CURIA *et al*., 2008; RAPACZ *et al*., 2016), o que não os tornam menos importantes.

Esses efeitos da GD no modelo de pilocarpina podem estar relacionados com um conjunto de fatores, como por exemplo, a ação de GD em inibir glutamato (PEREIRA *et al*, 1997) uma vez que neste modelo verificou-se um aumento dos níveis de glutamato no hipocampo de roedores (SMOLDERS *et al*., 1997). Outro mecanismo que pode estar envolvido é a possível ação de GD em modular a enzima GAD, o que levaria a um aumento de GABA, como demonstrado no modelo de isoniazida (dados não publicados do laboratório). E por fim, esses efeitos poderiam estar relacionados com a atividade de GD em reduzir EROS, NO e citocinas pró-inflamatórias, visto que a neuroinflamação está envolvida em SE (REDDY e KURUBA, 2013; PFLUGER *et al*, 2016).

Tendo em vista que fármacos anticonvulsivantes usados na clínica, não atuam somente em um receptor específico e sim existem vários alvos moleculares (ROGAWSKI *et al*, 2016), resolveu-se investigar o efeito de GD nas doses de 100 e 300 mg\kg em camundongos, sobre mais quatro modelos de convulsões com mecanismos de ações distintos, a fim de encontrar um melhor entendimento do mecanismo de proteção de GD nas convulsões.

Segundo Rassendren e Audinat (2016) a adenosina é um importante modulador endógeno da excitabilidade neuronal, proporcionando efeitos anticonvulsivos tanto *in vivo* quanto *in vitro* e cada vez mais se tem estudado o envolvimento da sinalização purinérgica no tratamento de crises convulsivas e SE. A aminofilina, um antagonista de receptores adenosinérgicos, que também atua na enzima fosfodiesterase (JAISHREE *et al*., 2003), foi um dos modelos estudados e verificou-se que GD nas duas doses testadas aumentou a latência para primeira convulsão. Alguns estudos têm mostrado o possível envolvimento dos radicais livres (EROS-ERNS) e estresse oxidativo em convulsões induzidas por aminofilina (CHINDO *et al*., 2014). Sendo assim, esse estudo sugere que a proteção da GD pode estar relacionada tanto com o envolvimento na modulação adenosinérgica, quanto com a já demonstrada atividade de diminuir EROS devido a atividade anti-inflamatória comprovada, que por sua vez, inibe a morte celular via sinalização da família p38 MAPKS (PFLUGER *et al*., 2016).

No modelo de Isoniazida, ocorre inibição da enzima GAD, que possui um papel crucial na inibição do neurotransmissor GABA e consequentemente excitabilidade neuronal, proporcionando convulsões (CHINDO *et al*., 2014). GD, na dose de 300 mg\kg, foi capaz de aumentar o limiar para primeira convulsão e, consequentemente, diminuir a mortalidade dos camundongos. Supõe-se que o mecanismo envolvido nessa proteção pode estar relacionado com o envolvimento de GD na modulação da enzima GAD, porém, para confirmar essa hipótese será necessário realizar um ensaio com GD avaliando a expressão da enzima GAD.

Gama-decanolactona, na maior dose testada foi eficaz contra convulsões induzidas por 4-AP, tanto na redução da porcentagem de convulsões, quanto no aumento da latência para primeira convulsão. 4-AP é um potente bloqueador de canais de K^+, que estimula o aumento da absorção pré-sináptica de Ca^{2+}, ativando receptores NMDA e induzindo a excitabilidade (YOW *et al*., 2013). Esse evento nos revela que a proteção de GD pode estar relacionada à modulação dos canais de K^+. O aumento da neurotransmissão gabaérgica, como mencionado acima, no modelo de isoniazida, também pode resultar na condutância de K^+, deste modo, a GD poderia estar agindo de forma direta ou indiretamente nos canais de K^+ (MANTE *et al*., 2013).

Em contrapartida a GD não protegeu contra convulsões induzidas por picrotoxina, que é um antagonista dos receptores $GABA_{A,}$ o que se pode inferir que a GD não tem efeito direto nos receptores específicos $GABA_A$ (CHINDO *et al*., 2014).

A GD na dose de 300 mg\kg diminuiu a coordenação motora de camundongos no teste de rotarod, nos primeiros trinta minutos observados. No tempo de 60 minutos, os camundongos voltaram a caminhar normalmente pela haste, indicando uma leve atividade sedativa, essa atividade corrobora aos resultados do teste feito para indução de sono por DZP, na qual GD também na dose de 300mg\kg obteve uma ação sedativa. Estes resultados reforçam outros estudos que demonstram que GD aumentou o sono induzido por pentobarbital, na mesma proporção que o controle DZP 2 mg\kg (COELHO DE SOUZA *et al*., 1997). Visto que fármacos anticonvulsivantes utilizados atualmente na clínica, muitas vezes também apresentam atividade sedativa, reduzindo assim o número e intensidade de crises convulsivas, a leve atividade sedativa de GD, não seria visto como problema e sim como um dos mecanismos envolvidos na atividade anticonvulsivante (KAMINSKI *et al*., 2014; HABIBI *et al*., 2016).

7 CONCLUSÃO

Após o conjunto de dados gerados neste trabalho, pode-se concluir que a atividade anticonvulsivante da GD observada em alguns dos modelos estudados, deve-se, a modulação de receptores de adenosina e canais de K^+, o que explica também a leve atividade sedativa observada. Esses parâmetros podem estar relacionados com a já comprovada atividade de GD, como antagonista glutamatérgica.

Nos resultados obtidos *in vitro* e na atividade de GD nos parâmetros bioquímicos observados, conclui-se que a redução de estresse oxidativo, dano ao DNA e morte neuronal, muito possivelmente sejam pela atividade da GD na redução da inflamação e consequente redução da morte neuronal via inibição da p-38 MAPKs.

Moléculas com potencial anti-inflamatótio, têm sido bem propostos para tratamento de crises convulsivas e dano neuronal. Sendo assim, GD se torna um novo alvo com potencial terapêutico para desordens do SNC, mais especificamente convulsões, o que torna necessário o desenvolvimento de novos estudos para melhor elucidar o seu mecanismo de ação.

8 PERSPECTIVAS

Avaliar o efeito de gama-decanolactona:

- sobre convulsões induzidas por PTZ no modelo de Kindling, usando os antagonistas adenosinérgicos no final do tratamento;
- na modulação do sistema GABAérgico;
- sobre a expressão de proteínas GAD65, GABAA, GABA-T;
- sobre parâmetros bioquímicos: peroxidação lipídica, nitrito e nitrato, radicais livres, superóxido dismutase, glutationa peroxidase, catalase;
- sobre a função mitocondrial (complexo I, II e biossíntese de ATP);
- sobre parâmetros bioquímicos: em córtex e hipocampo retirados dos modelos de 4-AP, aminofilina e isoniazida.

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9 ANEXOS

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 28513
Título: PARÂMETROS COMPORTAMENTAIS E NEUROTÓXICOS DE GAMA-DECANOLACTONA EM MODELOS EXPERIMENTAIS DE EPILEPSIA, ESTRESSE OXIDATIVO E MUTAGENICIDADE

Vigência: 01/03/2015 à 31/12/2016

Pesquisadores:

Equipe UFRGS:

PATRÍCIA PEREIRA - coordenador desde 01/03/2015
Pricila Fernandes Pfluger - Aluno de Mestrado desde 01/03/2015

Equipe Externa:

Jenifer Saffi - pesquisador desde 01/03/2015
Cassiana Viau - pesquisador desde 01/03/2015

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 15/06/2015 - Sala 330 - Prédio do Anexo I da Reitoria - Campus Centro - Porto Alegre - RS, em seus aspectos éticos e metodológicos, para a utilização de 320, camundongos Swiss, machos, de 60 dias, provenientes do CREAL, de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Quinta-Feira, 25 de Junho de 2015

CRISTIANE MATTE
Coordenador da comissão de ética