UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

ANÁLISE DE DANOS AO DNA NO HIPOCAMPO DE CAMUNDONGOS COM LÚPUS INDUZIDO POR PRISTANE SUPLEMENTADOS COM VITAMINA D

THAÍS EVELYN KARNOPP

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THAÍS EVELYN KARNOPP

Orientador: Prof. Dr. Odirlei André Monticielo
Coorientador: Prof. Dr. Alexandre Rieger

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Epígrafe:

“Cada sonho que você deixa para trás é um pedaço
do seu futuro que deixa de existir”
(Steve Jobs)

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RESUMO

Introdução: O lúpus eritematoso sistêmico (LES) é uma doença autoimune inflamatória crônica e multissistêmica. As manifestações da doença podem acometer o sistema nervoso central (SNC), sendo esta condição conhecida como lúpus neuropsiquiátrico (NPSLE). Na sua fisiopatogenia, há rompimento da barreira hematoencefálica (BHE) que propicia o surgimento de lesões e disfunções neuronais que podem se expressar como alterações cognitivas, distúrbios psiquiátricos, convulsões e cefaléia. O processo inflamatório contribui para o aumento do estresse oxidativo e danos ao DNA. Estudos demonstram que a vitamina D tem efeito imunomodulador e protetor em células do sistema imune e também no SNC, sendo capaz de atravessar a BHE devido a sua lipossolubilidade. O modelo animal de lúpus induzido por pristane (PIL) mimetiza manifestações clínicas e laboratoriais do LES em humanos. Apesar de apresentar vários sintomas da doença, o modelo é pouco estudado quanto aos mecanismos fisiopatogênicos de disfunção neuronal e suas repercussões neuropsiquiátricas.

Objetivo: Estudar a influência da suplementação da vitamina D no dano ao DNA de células do hipocampo em animais com lúpus induzido por pristane.

Metodologia: O modelo experimental foi induzido com uma única injeção intraperitoneal de 500ul de pristane em camundongos BALB/c fêmeas de 8-12 semanas de idade. Os animais foram divididos em três grupos: grupo controle (CO), grupo lúpus induzido por pristane (PIL) e grupo lúpus induzido por pristane suplementado com vitamina D (VD). A partir da indução do modelo, os animais do grupo VD foram tratados com vitamina D através de injeção subcutânea de 100ul de Calcijex [2ug/kg/animal] diluído em PBS-Tween 20 em dias alternados durante 180 dias. A eutanásia foi realizada após 180 dias e o cérebro foi coletado e dividido em hemisfério direito e hemisfério esquerdo para análises histológicas e de ensaio cometa, respectivamente.

Resultados: O grupo PIL apresentou maior infiltrado de IgG total no hipocampo quando comparado ao grupo CO ($p= 0,01$). A vitamina D demontrou uma tendência em reduzir o infiltrado de IgG, apesar de não ser estatisticamente significativo. O grupo VD apresentou numericamente um maior dano ao DNA quando comparado ao grupo PIL e CO. Não houve aumento na

expressão do receptor de vitamina D (VDR) no grupo tratado, os níveis de expressão da proteína se assemelharam nos grupos CO, PIL e VD. O tamanho do hipocampo não apresentou diferença entre os grupos. Houve correlação positiva entre a expressão de VDR e infiltrado de IgG no hipocampo r= 0,7585 (*p*= 0,0001).

Conclusão: O modelo PIL utilizado neste estudo apresentou maior infiltrado de IgG no hipocampo, o que pode representar disfunção da BHE e potencial risco para desenvolver NPSLE. Este achado torna possível a utilização deste modelo para avaliar aspectos fisiopatogênicos do NPSLE. A vitamina D não preveniu dano ao DNA das células do hipocampo. Estudos adicionais são necessários para o melhor entendimento do papel da vitamina D no NPSLE.

Palavras-chaves: Lúpus eritematoso sistêmico, Ensaio Cometa, Dano ao DNA, Lúpus Neuropsiquiátrico, Vitamina D.

ABSTRACT

Introduction: *Systemic lupus erythematosus (SLE) is a chronic multisystem inflammatory autoimmune disease. Manifestations of the disease can affect the central nervous system (CNS), and this condition is known as neuropsychiatric lupus (NPSLE). In its pathophysiology, there is disruption of the blood-brain barrier (BBB) that promotes the emergence of neuronal lesions and dysfunctions that can express themselves as cognitive changes, psychiatric disorders, seizures and headache. The inflammatory process contributes to increased oxidative stress and DNA damage. Studies show that vitamin D has immunomodulatory and protective effect in cells of the immune system and also in the CNS, being able to cross the BBB due to its liposolubility. The animal model of pristane-induced lupus (PIL) mimics clinical and laboratory manifestations of SLE in humans. Despite presenting several symptoms of the disease, the model is poorly studied regarding the pathophysiological mechanisms of neuronal dysfunction and its neuropsychiatric repercussions.*

Objective: *To study the influence of vitamin D supplementation on hippocampal cell DNA damage in animals with pristane-induced lupus.*

Methodology: *The experimental model was induced with an intraperitoneal injection of 500ul of pristane in 8-12 weeks old female BALB/c mice. The animals were divided into three groups: control group (CO), pristane-induced lupus group (PIL) and pristane-induced lupus group supplemented with vitamin D (VD). From the model induction, animals from the VD group were treated with vitamin D by subcutaneous injection of 100ul Calcijex [2ug / kg / animal] diluted in PBS-Tween 20 every other day for 180 days. Euthanasia was performed after 180 days and the brain was collected and divided into right hemisphere and left hemisphere for histological and comet assay analysis, respectively.*

Results: *The PIL group presented higher total IgG infiltrate in the hippocampus when compared to the CO group (p= 0.01). Vitamin D has shown a tendency to reduce IgG infiltrate, although it is not statistically significant. The VD group presented numerically greater DNA damage when compared to the PIL and CO group. There was no increase in VDR expression in the treated group, protein*

expression levels were similar in CO, PIL and VD groups. Hippocampal size did not differ between groups. There was a positive correlation between the expression of vitamin D receptor (VDR) and IgG infiltrate in the hippocampus r= 0.7585 (p= 0.0001).

Conclusion: *The PIL model used in this study showed a larger IgG infiltrate in the hippocampus, which may represent BBB dysfunction and potential risk for developing NPSLE. This finding makes it possible to use this model to evaluate pathophysiological aspects of NPSLE. Vitamin D did not prevent damage to hippocampal cell DNA. Further studies are needed to better understand the role of vitamin D in NPSLE.*

Keywords: *Systemic lupus erythematosus, Comet Assay, DNA Damage, Neuropsychiatric lupus, Vitamin D.*

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LISTA DE ABREVIATURAS E SIGLAS

1,25 (OH) D – 1,25 dihidroxivitamina D

ACR – Celégio americano de reumatologia (*American college of reumathology)*

ANA – Anticorpos antinucleares (*Antinuclear antibody)*

Anti-dsDNA – Anticorpos Anti-DNA de dupla hélice (*double strength DNA)*

Anti-ssDNA – Anticorpos Anti-DNA de hélice simples (*single strength DNA)*

Anti-Sm – Anticorpos Anti-Smith

BHE – Barreira hematoencefálica

BSA – Albumina do soro bovino (*Bovine serum albumin*)

DMSO – Dimetil sulfóxido (*Dimethyl sulfoxide)*

DNA – Ácido desoxirribonucleico (*Deoxyribonucleic acid)*

EC – Ensaio cometa

EDTA – Ácido etilenodiamino tetra-acético *(Ethylenediamine etraacetic acid)*

FAN – Fator antinuclear

FD – Frequência de dano

HE – Hematoxilina/Eosina

ID – Índice de dano

IFN I – Interferon do tipo 1

IFNα – Interferon alfa

IFNγ – Interferon gama

IgG – Imunoglobulina G

IL – Interleucina

IP – Injeção intraperitoneal

LCR – Liquido Cefalorraquidiano

LES – Lúpus Eritematoso Sistêmico

NaCl – Cloreto de Sódio

NaOH – Hidróxido de Sódio

NK – Células *Natural Killer*

NMDAR – Receptor Anti-metil-D-aspartato *(N-methyl-D-aspartate receptor)*

NPSLE – Lúpus Neuropsiquiátrico (*Neuropsychiatric Systemic Lupus Erythematosus)*

PBS – Tampão Fosfato Salino (*Phosphate-Buffered Saline*)

PIL – Lúpus Induzido por Pristane (P*ristane-induced Lupus)*

RNA – Ácido Ribonucleico *(Ribonucleic acid)*

ROS – Espécies Reativas de Oxigênio (*Oxygen Reactive Species*)

SNC – Sistema Nervoso Central

SNP – Sistema Nervoso Periférico

SLICC - *Systemic Lupus International Collaborating Clinics*

SPSS - Pacote Estatístico para as Ciências Sociais (*Statistical Package for the Social Sciences)*

TCA – Ácido Tricloroacético *(Trichloroacetic acid)*

TLR – Receptores do tipo Toll (*Toll like receptors*)

TNFα – Fator de Necrose Tumoral alfa (*Tumor Necrosis Factor Alpha)*

T_{reg} – Células T reguladoras

VDR – Receptor de Vitamina D (*Vitamin D Receptor)*

$ZnSO_4$ – Sulfato de Zinco

1. INTRODUÇÃO

O lúpus eritematoso sistêmico (LES) é uma doença autoimune inflamatória crônica com envolvimento multissistêmico. Caracteriza-se por uma alteração da homeostase imunológica, que envolve a ativação anormal de células T, células B e células apresentadoras de antígenos, levando a produção de autoanticorpos e deposição de imunocomplexos (1–3). A etiologia da doença é pouco conhecida, porém sabe-se da participação de fatores hormonais, ambientais, genéticos e imunológicos (4). O LES atinge principalmente mulheres, entre 20-45 anos, com uma proporção de acometimento de 9:1 em relação aos homens. No Brasil, a estimativa é que existam em torno de 65.000 pessoas acometidas por esta doença (5). As manifestações clínicas são amplas e podem causar lesões de pele, alterações no sistema musculoesquelético, nos aparelhos cardiocirculatório e pulmonar, nos rins, nos elementos do sangue e também no sistema nervoso central (SNC) (5,6).

As manifestações do SNC do LES (lúpus neuropsiquiátrico, NPSLE) estão presentes em mais de 50% dos casos. A diversidade de sintomas, por vezes desconhecidos ou confundidos com desordens comuns, torna difícil estabelecer um diagnóstico ou tratamento. Devido à dificuldade no diagnóstico, os dados de prevalência do NPSLE não são bem estabelecidos, tendo uma grande variabilidade entre os estudos (de 10% a 80%) (7–9). Embora a patogênese do NPSLE ainda não tenha sido bem caracterizada, acredita-se que citocinas pró-inflamatórias promovam a disruptura da barreira hematoencefálica (BHE). As ações fisiológicas, patológicas ou traumas, também podem levar a este fenômeno. Com o comprometimento da BHE, células do sistema imune e autoanticorpos podem atravessá-la, promovendo o surgimento de um ambiente inflamatório com ativação da glia, neurodegeneração e consequentes resultados clínicos como disfunção cognitiva, psicose, transtornos de humor, entre outros (10,11).

A inflamação resultante dos períodos de exacerbação de atividade de doença desencadeia aumento descontrolado na geração de radicais livres, que excede a capacidade antioxidante do organismo, levando a um ambiente de estresse oxidativo. Este estresse causa danos às proteínas, lipídeos e ao DNA

e contribui com a inflamação crônica, tornando-se assim, um ciclo vicioso (12–15).

Terapias alternativas têm sido investigadas para controlar as manifestações clínicas do LES, como por exemplo, a vitamina D, que atua na imunomodulação das respostas imunes inata e adaptativa. Estudos publicados nos últimos anos demonstram a importância da vitamina D, não apenas no metabolismo ósseo e do cálcio, mas também na regulação do sistema imune e em outros tecidos, como o cérebro (16–18). Além disso, a deficiência desta vitamina está associada à gravidade das manifestações clinicas em doenças autoimunes, como o LES (17,18).

Além de atuar na imunomodulação a vitamina D têm receptores em diversos tecidos (19). Algumas áreas cerebrais expressam o receptor de vitamina D (*Vitamin D Receptor*, VDR), tais como cerebelo, tálamo, hipotálamo, gânglios basais, hipocampo, sistema olfativo e os córtices temporais, orbitários e cingulados. Estudos indicam que a vitamina D tem efeito neuroprotetor além de estar relacionada com o desenvolvimento cerebral fetal e de proteção contra danos ao DNA (20–25).

Pesquisar NPSLE em humanos é difícil devido à grande heterogeneidade da doença e à dificuldade em se ter acesso ao SNC. Os modelos animais têm sido um ótimo recurso para novas descobertas, tanto para entender melhor a fisiopatogenia da doença quanto para descoberta de novos tratamentos (26,27).

Sabe-se que o modelo de lúpus induzido por pristane (PIL) é o modelo animal que desenvolve a maior gama de manifestações do LES. Entretanto, são raros os estudos com este modelo que demonstrem as implicações da doença no SNC, bem como o efeito da administração de vitamina D sobre estas manifestações.

2. REVISÃO SISTEMÁTICA DA LITERATURA

2.1 Estratégias para localizar e selecionar as informações

A estratégia de busca envolveu as seguintes bases de dados: PubMed e LILACS. Foram incuídos artigos de 1978 a 2019. Foram realizadas buscas através da lista de termos e suas combinações: “vitamin d”, “lupus”, “systemic lupus erythematosus”, “vitamin d and lupus”, “neuropsychiatric lupus”, “vitamin d and brain”, “DNA damage in lupus”, “comet assay” “animal model for lupus”, “animal model for NPSLE”, “Mice, Inbred MRL lpr”.

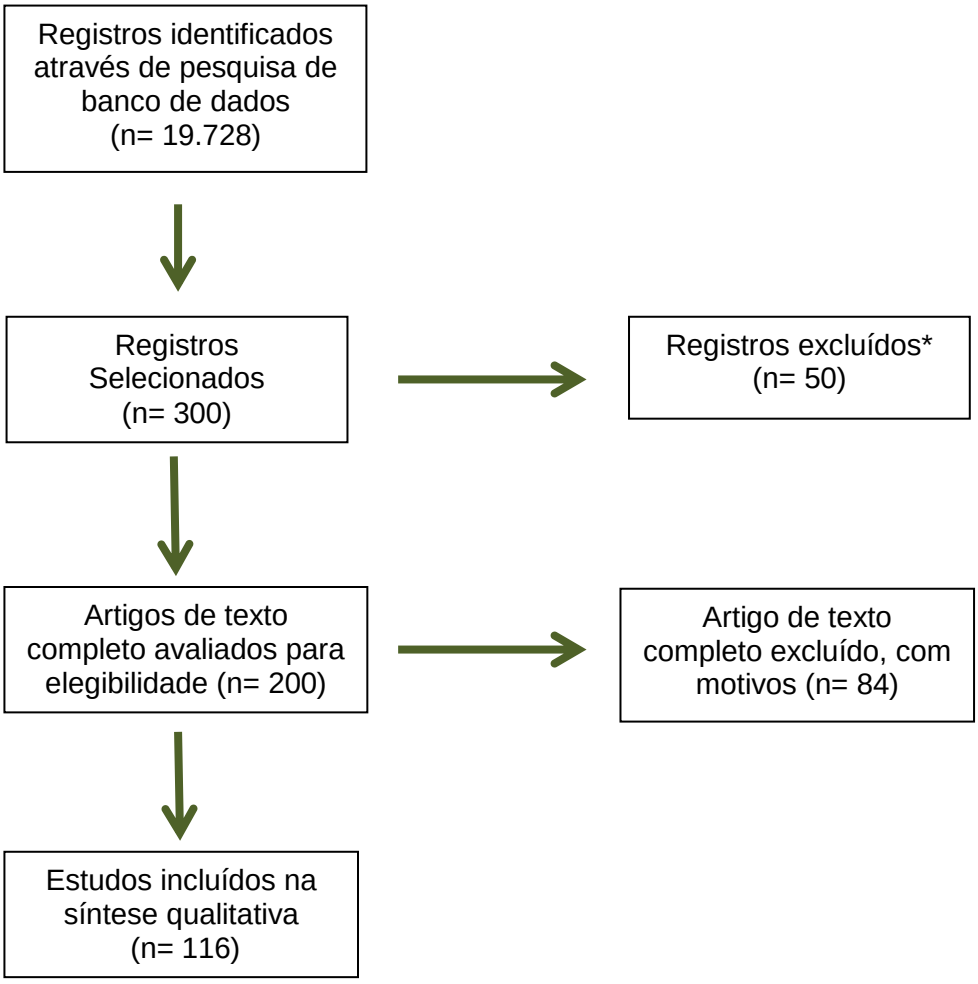

Figura 1. Modelo esquemático da estratégia de busca de informações. *Critérios de exclusão dos artigos: tema não relacionado aos objetivos da pesquisa; artigos não disponíveis na íntegra; artigos não disponíveis em inglês e/ou português.

2.2 Lúpus Eritematoso Sistêmico (LES)

O conceito chave para a patogenia do LES é o desequilíbrio entre a produção de células apoptóticas (células com morte programada) e a disposição de material apoptótico. Os *debris* apoptóticos, em geral, são retirados rapidamente do organismo e não são acessíveis ao sistema imune. Entretanto, quando há falhas e permanência de alguns *debris* contendo ácidos nucleicos, pode-se estimular uma resposta inflamatória através da ativação de receptores de reconhecimento de ácidos nucleicos como, por exemplo, os receptores do tipo Toll (TLR) (28).

O LES é uma doença inflamatória crônica, caracterizada pela produção de autoanticorpos e lesões em diversos órgãos e tecidos, é considerada imprevisível, remitente e recorrente (29). Os sintomas surgem ao longo do desenvolvimento da doença e devido à grande diversidade destes, em 1982, o *American College of Rheumatology (ACR)* estabeleceu 11 critérios para diagnóstico de LES. Tais critérios foram revisados e reestabelecidos pelo *Systemic Lupus International Collaborating Clinics (SLICC),* em 2012. Para a obtenção de um diagnóstico de lúpus, o paciente deve apresentar quatro ou mais sintomas (sendo no mínimo 1 critério clínico e 1 critério laboratorial/ imunológico) (tabela 1) (5,30–32).

Danos a órgãos nobres e infecções são as principais causas de morte de pacientes lúpicos, juntamente com complicações cardiovasculares. Os pacientes também podem apresentar anemia, fadiga, dores e edema nas articulações, alopecia, eritemas cutâneos e febre. Pacientes com LES podem apresentar manifestações neurológicas quando o sistema nervoso é afetado. Os sintomas podem desenvolver-se repentinamente ou em períodos de remissão e recidiva ao longo do tempo, podendo variar de acordo com a localização e extensão da lesão (6).

Tabela 1 - Critérios para diagnóstico de Lúpus, conforme *Systemic Lupus International Collaborating Clinics - SLICC*.

Critérios Clínicos	Critérios Imunológicos
Lúpus cutâneo agudo	ANA/FAN – Anticorpos/Fator Antinuclear
Lúpus cutâneo crônico	Anti-DNA
Úlceras nasais e/ou orais	Anti-Sm
Alopecia não transportadora	Anticorpos Antifosfolipídios
Artrite	Baixo complemento (C3; C4; CH50)
Serosite	Teste de Coombs direto
Complicações renais	
Complicações neurológicas	
Anemia hemolítica	
Leucopenia	
Trombocitopenia	

Fonte: Adaptado de PETRI et al., 2012 (32).

2.3 Lúpus Neuropsiquiátrico (NPSLE)

O lúpus neuropsiquiátrico (NPSLE) engloba um conjunto de manifestações clínicas que envolve o SNC e o sistema nervoso periférico (SNP) (33). Estudos apontam uma variação de 10-80% de prevalência do NPSLE em portadores de LES (7–9). Em meta-análise que incluiu 10 estudos, foi relatado cerca de 56% de acometimento neuropsiquiátrico entre os indivíduos com LES (8).

Devido à diversidade de sintomas, o ACR (1999) (34) descreveu 19 critérios, divididos em manifestações centrais e periféricas (Tabela 2), para estabelecer um quadro clínico do lúpus neuropsiquiátrico, entretanto, os critérios são inespecíficos para o estabelecimento de um diagnóstico.

O LES é capaz de produzir uma elevada gama de autoanticorpos. Até o momento, foram descritos cerca de 180 tipos de autoanticorpos em soro de pacientes lúpicos. De maneira geral, o cérebro é protegido pela BHE dos autoanticorpos circulantes. Entretanto, se a barreira perder sua integridade, os autoanticorpos podem ter acesso ao parênquima cerebral (35).

Tabela 2. Síndromes neuropsiquiátricas observadas em portadores de Lúpus Eritematoso Sistêmico

Sistema Nervoso Central	Sistema Nervoso Periférico
Meningite asséptica	Poliradiculoneiropatia desmielinizante
Doença cerebrovascular	inflamatória aguda (Síndrome de Guillain-Barre)
Síndrome desmielinizante	Transtorno autonômico
Dor de cabeça (incluindo enxaqueca e	Mononeuropatia única ou multiplexada
hipertensão intracraniana benigna)	Miastenia grave
Transtorno do movimento (coreia)	Neuropatia craniana
Mielopatia	Plexopatia
Distúrbios convulsivos	Polineuropatia
Estado confuso agudo	
Transtorno de ansiedade	
Disfunção cognitiva	
Transtorno do humor	
Psicose	

Fonte: Adaptado de LIANG et al., 1999 (34).

Jafri e colaboradores (11) descreveram um modelo de lesão no NSPLE, desenvolvido em 3 etapas. Inicialmente, a periferia produz os anticorpos antineuronais. Logo após, ocorre uma violação da BHE, que pode ou não estar relacionada ao LES, seguida de danos neuronais mediados por anticorpos.

Nesse sentido, a integridade da BHE pode ser prejudicada por infecções bacterianas, trauma e isquemia cerebral (36). Tem-se demonstrado, através de estudos com roedores, que a disruptura da BHE ocorre de forma regional, de acordo com a injúria. Estes estudos demonstram que, após a estimulação bacteriana de lipopolisacarídeos, existe um influxo de IgG apenas no hipocampo e que a administração sistêmica de adrenalina permite a passagem de IgG para a amígdala (37–39).

Com isso, pode-se inferir que o efeito de um potencial anticorpo antineuronal depende da localização da violação da BHE, e este depende da injúria provocada, alterando assim a variação das manifestações observadas no NPSLE (11).

2.4 Barreira Hematoencefálica (BHE) e Disfunção

Em geral a BHE forma uma interface entre o sangue e o cérebro, protegendo assim o cérebro dos componentes sanguíneos como anticorpos, citocinas, células imune, entre outros (40). Esta barreira é composta de células endoteliais, astrócitos, pericitos, macrófagos perivasculares e membrana. A BHE permite a passagem livre de algumas moléculas essenciais solúveis em água, enquanto outros nutrientes complexos dependem de sistemas de transporte altamente seletivos para entrar no cérebro (41).

Alterações na formação celular da barreira ou uma ruptura nas suas junções podem causar um aumento de permeabilidade, resultando no influxo de moléculas pró-inflamatórias, células e autoanticorpos que perturbam a função do cérebro e levam a um dano neuronal (figura 2) (42). Tem sido demonstrado que quantidades excessivas de neurotransmissores, citocinas, quimiocinas e hormônios periféricos podem influenciar na permeabilidade da BHE (40).

Recentemente, foi demonstrado que alterações no líquido cefalorraquidiano (LCR) podem ativar gatilhos que levam ao NPSLE (43). Os autores inferem que a disfunção na barreira LCR-sangue facilita a exposição do cérebro aos anticorpos e entrada anormal de linfócitos nos ventrículos cerebrais. Têm sido relacionados pelo menos 20 autoanticorpos, no soro e no LCR, ao NPSLE, estes podem atravessar a barreira, quando sua integridade está comprometida (37,44–46).

O gene *Trex1* está associado à autoinflamação mediada por IFNα devido à deposição de ácido nucleico intracelular. Uma mutação neste gene pode levar a ruptura na BHE (47,48). Estudos têm demostrado um grave envolvimento do SNC quando presente o polimorfismo neste gene (49,50). Além do IFNα, uma variedade de citocinas têm sido identificadas como mediadores inflamatórios. Essas citocinas, tais como TNFα, IL-1, IL-6 e IFNγ, têm um papel patogênico e podem levar a disfunção da BHE (33,51–53).

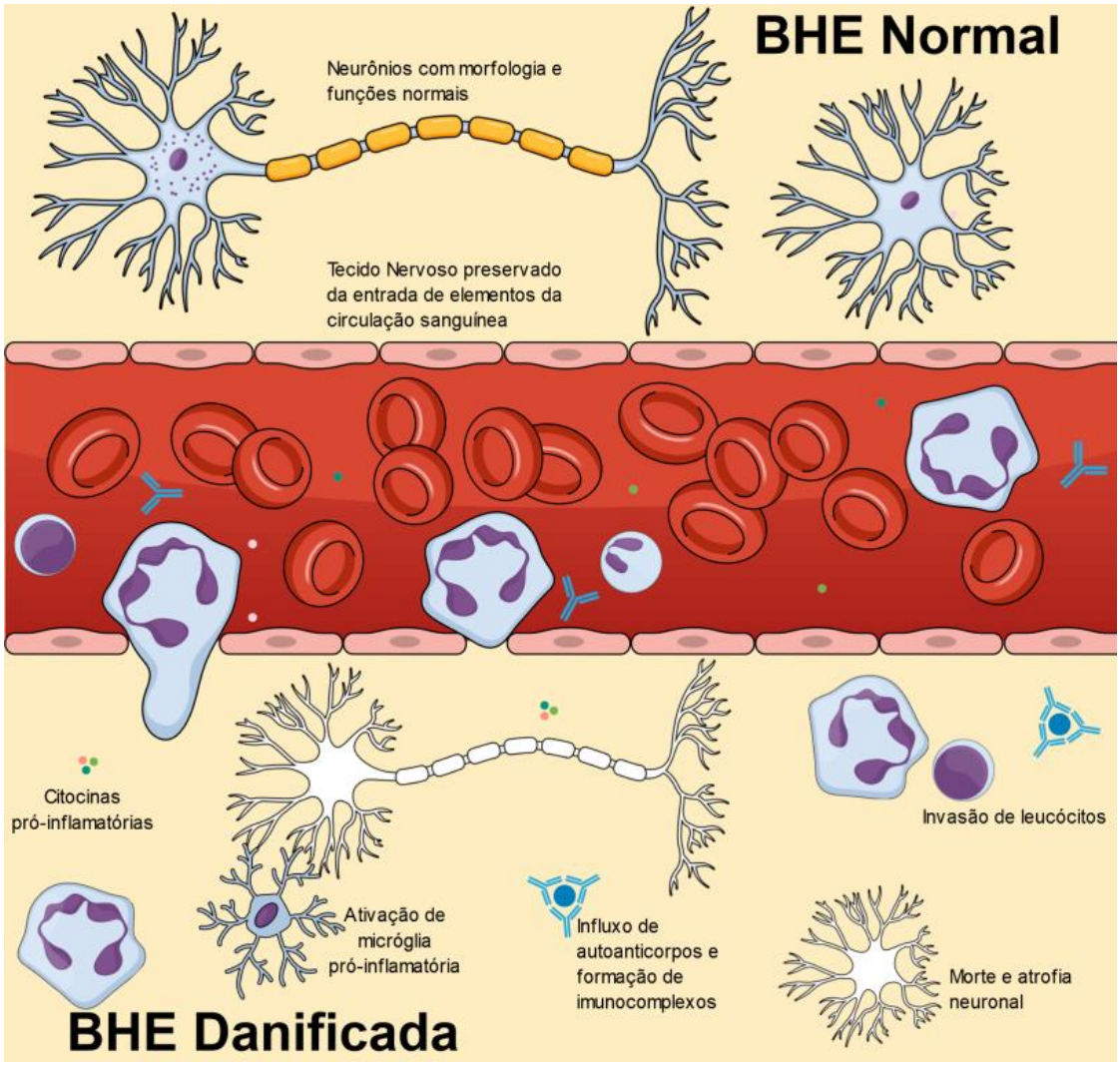

Figura 2. A barreira hematoencefálica (BHE), estrutura e disfunção. A BHE impede que alguns componentes celulares e inflamatórios cheguem ao cérebro. Quando rompida por algum trauma ou infecção, autoanticorpos, citocinas inflamatórias, imunocomplexos e leucócitos invadem o cérebro e se inicia o processo inflamatório nesta região. Devido às injurias e inflamação, ocorre a morte neuronal, complicações cognitivas, disfunção de memória e transtornos causados no NPSLE. Fonte: Autor.

Os autoanticorpos também podem levar a uma disfunção na BHE. O receptor anti-metil-D-aspartato (anti-NMDAR) e anti-ribosomal-P são anticorpos neuropáticos circulantes e não são capazes de cruzar a BHE, entretanto, têm sido reportados como efetores no NPSLE (54,55). O potencial efeito antineuronal do anticorpo vai depender da localização da infecção da BHE, dependendo também da lesão induzida. Estas diferenças podem levar a uma variação das manifestações observadas no NPSLE (11).

2.5 Vitamina D

A vitamina D pode ser encontrada em diversas formas, sendo a vitamina D3 ou colecalciferol e a vitamina D2 ou ergocalciferol, os principais tipos encontrados. A primeira é formada na pele através da exposição à luz solar ou à luz ultravioleta (figura 3), além de fontes nutritivas como peixes gordurosos. A segunda é obtida através de alimentos de origem vegetal. Algumas limitações são encontradas para a síntese desta vitamina como, por exemplo, a idade, pigmentação da pele, uso de protetor solar e roupas (56).

Sabe-se que a vitamina D é importante no processo de absorção de cálcio dependente de vitamina. Sua forma ativa 1,25 dihidroxivitamina D [$1,25(OH)_2D$] tem por principal efeito aumentar a absorção de cálcio a partir do intestino. Este processo auxilia no aumento da mineralização osteóide, estimula a função dos osteoblastos e osteoclastos e suprime a secreção do hormônio da paratireoide (17,56).

Algumas funções não clássicas da vitamina D têm sido descritas, como efeitos sobre a proliferação e diferenciação celular, além de ações imunorregulatórias, resultando na capacidade de manter a tolerância e promover a imunidade protetora. Tanto as células apresentadoras de antígenos (macrófagos e células dendríticas) quanto as células T e B possuem a maquinaria necessária para sintetizar e responder a $1,25(OH)_2D$ (57).

Vários estudos relacionam a deficiência de vitamina D com a fisiopatogenia de doenças autoimunes, tais como esclerose múltipla, artrite reumatoide, diabete *mellitus* e LES. Um estudo realizado com 161 pacientes com doença do tecido conjuntivo não diferenciada demonstrou que, após dois anos de acompanhamento, os pacientes que evoluíram para doença definida tinham uma deficiência de vitamina D maior do que aqueles que permaneceram sem definição do quadro (58,59).

No sistema imune, a vitamina D atua na inibição da proliferação e bloqueia a diferenciação de células B e secreção de imunoglobulinas (60,61). Também suprime a proliferação de células T, auxilia na inibição da produção de citocinas inflamatórias como as interleucinas 17 e 21 (IL-17 e IL-21), aumentando a produção de citocinas anti-inflamatórias, como a IL-10 (17,62,63). Auxilia na inibição da diferenciação e maturação de células dendríticas (DC). Quando maduras, as DCs apresentam o antígeno a células T, facilitando uma resposta

imune contra esse antígeno. Quando imaturas, a apresentação do antígeno facilita a auto-tolerância (17). Assim a vitamina D desempenha um papel importante na regulação da auto-tolerância.

2.6 Vitamina D e Lúpus

Sabe-se que além do papel clássico da vitamina D, ela também atua na prevenção do câncer e nos efeitos intrácrinos de células imunes. A presença do VDR em células imunes sugere que esta vitamina pode mediar a comunicação entre as vias do sistema imunológico (inata e adaptativa) (58).

A $1,25(OH)_2D$ diminui a atividade de apresentação de antígenos, inibe a diferenciação de monócitos em células dendríticas, proliferação de células B, diferenciação de células plasmáticas, produção de anticorpos, além de atuar na síntese de diversas citocinas, como a IL-1, IL-6, IL-12 e TNFα, induz a ativação de células T reguladoras (T_{reg}) e células *natural killer* (NK), aumenta a apoptose induzidas por células dendríticas e linfócitos T (figura 3) (64).

Estudos têm demonstrado que em doenças autoimunes, a hipovitaminose por vitamina D é frequente, e diversas doenças apresentam essa deficiência, como por exemplo, artrite reumatoide, diabetes *mellitus* tipo 1 e LES (65–67). Em modelos animais de LES, a suplementação de vitamina D melhora a longevidade, reduz a proteinúria, diminui a artrite e previne lesões dermatológicas (58,68).

Em pacientes com LES em alta atividade foram relatados baixos níveis de vitamina D. Nos pacientes com baixa atividade da doença, os níveis da vitamina se mostraram mais elevados (69,70). Além da gravidade da doença, os níveis da vitamina D parecem ter associação inversamente proporcional a algumas comorbidades, como doenças cardiovasculares, necrose avascular, distúrbios do sono e fadiga (71–75). Os baixos níveis de vitamina D no sistema também podem estar associados ao aumento de estresse oxidativo, o que leva a lesões e quebras em proteínas e no DNA, inferindo que a vitamina possa ter um efeito protetor contra tais danos (76).

A vitamina D expressa metabólitos que são capazes de atravessar a BHE. Esta vitamina tem um papel importante na promoção de sobrevivência do neurônio. Pode suprimir as vias oxidativas no cérebro pela diminuição da formação de radicais livres de oxigênio (77). No cérebro humano e em murinos,

também são expressas proteínas VDR, em diversas regiões como no cerebelo, tálamo, hipotálamo, gânglios basais, hipocampo, sistema olfativo e os córtices temporais, orbitais e cingulados (21,22,78).

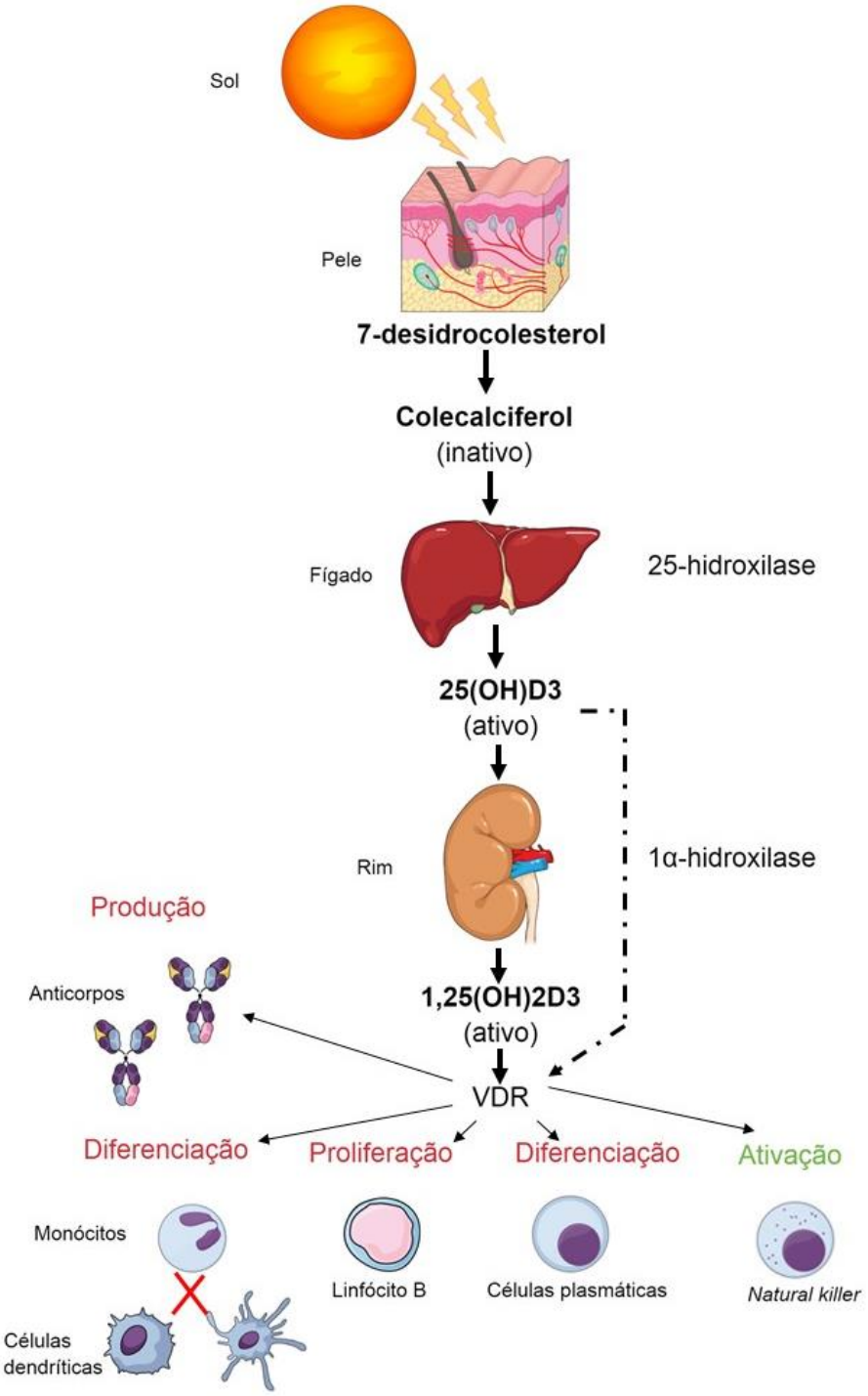

Figura 3. Metabolismo da vitamina D, ativação e efeitos imunomodulatórios. A vitamina D (colecalciferol) pode ser obtida a partir da ingestão alimentar ou por síntese na pele do 7-desidroxicolesterol em resposta à luz ultravioleta (UV) também emitidos pelos raios solares. No fígado, ocorre a hidroxilação por 25-hidroxilase, formando a 25-hidroxivitamina D (25(OH)D_3).

Nos rins, a 1α-hidroxilase atua na hidroxilação da $25(OH)D_3$ para a forma biologicamente mais ativa da vitamina D, a 1,25-di-hidroxivitamina D3 ($1,25(OH)_2D_3$). Tanto o $1,25(OH)_2D_3$ quanto o $25(OH)D_3$ são imunomoduladores por ligação a um receptor de vitamina D (VDR) presente no núcleo de quase todas as células imunológicas. Seus efeitos imunomoduladores incluem a inibição da diferenciação de monócitos em células dendríticas, a proliferação de células B, a diferenciação de células plasmáticas e produção de anticorpos. A vitamina também induz a ativação de células *natural killer* (NK). Fonte: Autor

2.7 Modelo animal de Lúpus Neuropsiquiátrico

Nos últimos anos vem crescendo estudos com animais que buscam compreender melhor as manifestações neuropsiquiátricas do NPSLE (26,27). A maioria dos modelos animais para lúpus produzem autoanticorpos, imunocomplexos e desenvolvem glomerulonefrite. Os modelos variam entre os animais que desenvolvem a doença espontaneamente como os modelos MRL/*lpr* e NZB/W F1, e os modelos animais induzidos, como por exemplo o lúpus induzido por pristane (BALB/c e C57BL/6J) (79,80).

Algumas pesquisas realizadas com camundongos MRL/*lpr* mostraram que estes animais apresentam disfunções cognitivas, depressão e ansiedade e depósito aumentado de IgG no cérebro (81–83). O modelo de lúpus induzido por pristane (PIL) é capaz de produzir uma ampla variedade de autoanticorpos específicos associados ao LES. Animais com PIL desenvolvem diversas manifestações semelhantes ao LES em humanos, tais como glomerulonefrite, aumento da expressão de TNFα, serosite e artrite (84).

2.8 Lúpus Induzido por Pristane (PIL)

O PIL é um modelo de lúpus experimental reproduzido em camundongos BALB/c ou C57BL/6J, conhecido por ser o modelo mais próximo do lúpus em humanos, quando comparados aos modelos espontâneos (85). A indução da doença é feita através de uma injeção intraperitoneal de 500µL de pristane, onde a solução oleosa provoca uma resposta imune (85). Este modelo é caracterizado pela produção de autoanticorpos específicos do LES, proteinúria, serosite, glomerulonefrite, aumento da expressão de TNFα e artrite (84,86,87).

A produção de citocinas inflamatórias têm um papel importante no PIL. O estímulo para a formação de autoanticorpos é feito pelas citocinas IL-6, IL-12, IFN I e IFNγ neste modelo (88,89). Estas citocinas promovem a produção de

autoanticorpos como anti-dsDNA, anti-ssDNA, anti-ribossomal P, ANA, anti-Sm e anti-Su (86,90–93).

O modelo PIL expressa grandes quantidades de IFN I e IFNγ, além de uma forte assinatura de IFN, característica da doença em pacientes com LES (87,94,95). Enquanto o IFN I é detectável em apenas duas semanas após a indução do pristane, os autoanticorpos aparecem apenas três meses após a indução da doença (96).

Citocinas proinflamatórias, como a IL-6 e IL-1 podem induzir o comprometimento da aprendizagem cognitiva, emocional e espacial. Camundongos com PIL têm uma expressão elevada de IL-6 no soro (87,95), também apresentam hipergamaglobulinemia semelhante ao LES em humanos (97).

Jeltsch-David e Muller (83), apontam que não há descrições de manifestações neuropsiquiátricas em animais com lúpus induzido. No entanto, camundongos BALB/c com PIL tendem a diminuir sua atividade de locomoção espontânea (98). Essa alteração pode estar associada à depressão, como já descrito em camundongos MRL/*lpr* e em pacientes lúpicos (82). Recentemente, foi descrita uma baixa regulação das subunidades NR2A do aminoácido NMDA no hipocampo. Este medeia certas formas de plasticidade sináptica e aprendizado, relacionadas ao comprometimento cognitivo no modelo PIL. Os autores demonstraram que o pristane pode ter um papel potencial em perturbar a BHE produzindo anticorpos anti-Sm. Eles também encontraram distúrbios de aprendizagem e memória, associados à baixa regulação da NR2A (99).

Sabe-se que em doenças autoimunes há envolvimento de espécies reativas de oxigênio (ROS). Quando em grande quantidade causam danos a proteínas e ao DNA. Recentemente, tem-se especulado o papel do ROS na patogênese do LES (100,101). Shaheen e colaboradores (85), demonstraram um aumento destas moléculas e do estresse oxidativo no peritônio de camundongos BALB/c com PIL. Os autores ainda inferem sobre esse aumento ter um papel central na patogenia da doença.

2.9 Ensaio Cometa

O ensaio cometa é uma ferramenta importante em estudos de biomonitoramento para avaliar a presença de danos no DNA induzidos por agentes alquilantes, intercalantes e oxidantes durante a exposição à xenobióticos (102). É considerado um teste simples, rápido e barato, comparado a outros testes, além de ser uma poderosa ferramenta para estudar fatores mutagênicos e carcinogênicos.

O ensaio cometa alcalino é utilizado para avaliar os níveis de quebra da hélice de DNA em células eucarióticas. As células são suspensas com agarose e submetidas a lise celular, com alta concentração de sal, o que auxilia na formação do nucleóide contendo *loops* de DNA enrolados, ligados à matriz. A eletroforese em pH alto permite a migração dos fragmentos de DNA e resulta em uma estrutura parecida com cometas, assim a intensidade da cauda, com DNA fragmentado, em relação à cabeça, parte do DNA íntegro, reflete o número de quebras do DNA. Há diversos tipos de ensaio cometa, como o neutro, alcalino e cinética de reparação (103).

O aumento de estresse oxidativo pode causar lesões no DNA que podem ser marcadores úteis em doenças crônicas. Alguns estudos relacionam o aumento do estresse oxidativo com a perda da autotolerância e consequente desenvolvimento da autoimunidade (100,101). Poucos estudos foram realizados utilizando o ensaio cometa em pacientes com LES. Estes demonstram um aumento do nível de dano ao DNA em linfócitos dos pacientes, além de uma deficiência na capacidade de reparação dos mesmos (104–106).

3. MARCO CONCEITUAL

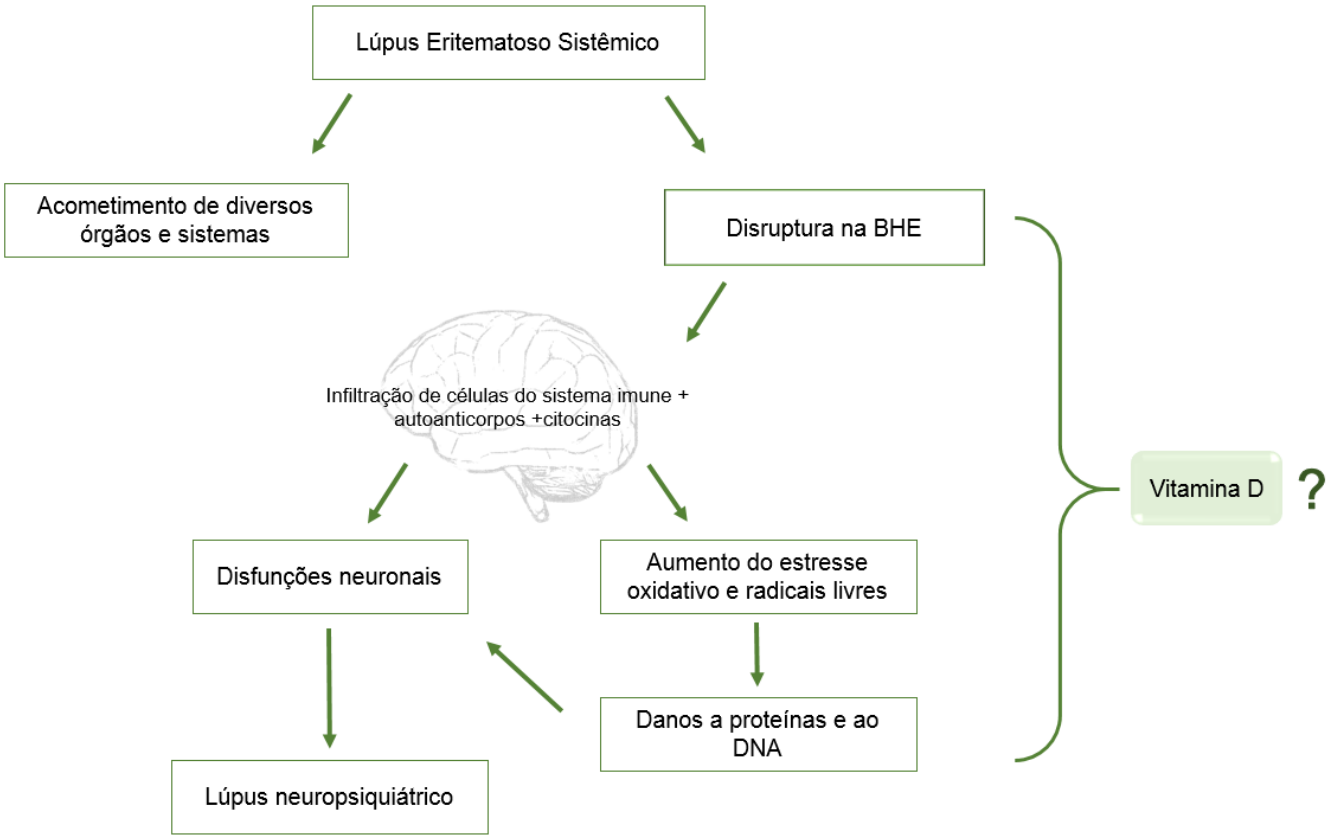

Figura 4. Marco conceitual do Lúpus Neuropsiquiátrico

A geração de autoanticorpos contra constituintes celulares do próprio hospedeiro leva à inflamação crônica que acomete diversos órgãos e sistemas do corpo. Quando atinge o SNC desencadeando quebra na BHE, há infiltração de células do sistema imune, como linfócitos T e B, citocinas e autoanticorpos. Esse aumento descontrolado da inflamação, gera aumento de radicais livres e estresse oxidativo no local do acometimento, podendo levar à danos no DNA e em proteínas. Esta sequência de eventos pode levar ao comprometimento das funções do SNC, diversos sintomas podem ser apresentados, como disfunções cognitivas, convulsões, distúrbios de humor, depressão e outros. Estes eventos caracterizam o Lúpus Neuropsiquiátrico. A vitamina D possui diversas funções fisiológicas, principalmente no metabolismo ósseo e do cálcio, no entanto, recentemente, descobriu-se efeito no metabolismo do sistema endócrino e na imunorregulação. Ainda, a vitamina D é capaz de diminuir o estresse oxidativo e proporcionar proteção ao sistema neuronal. No entanto, até o momento, não

existem estudos descrevendo tais efeitos relacionados ao NPSLE e o potencial papel da vitamina D em reduzir danos.

4. JUSTIFICATIVA

No LES, os autoanticorpos são dirigidos contra os antígenos nucleares, incluindo DNA, RNA e proteínas ligadoras de ácido nucleico. Há inflamação crônica, que pode levar a danos ao DNA. Além da inflamação, complicações neuropsiquiátricas podem se manifestar a partir da disruptura da barreira hematoencefálica e gerar danos em células neuronais. Terapias alternativas têm sido exploradas, como a vitamina D, que tem apresentado efeito protetor contra danos ao DNA. Além disso, os efeitos adversos desta suplementação são menores do que outros tratamentos, pois essa molécula é produzida pelo próprio organismo. Entretanto, há carência de dados na literatura referente ao NPSLE e possíveis danos ao DNA e o papel da vitmaina D neste contexto.

A questão de pesquisa deste projeto está em explorar e compreender as características fisiopatogênicas do NPSLE em modelo experimental de lúpus induzido por pristane submetido ao tratamento com vitamina D, identificando os danos ao DNA que podem estar relacionados com o aumento da inflamação e a disfunção da BHE.

5. OBJETIVOS

5.1Objetivo Principal

- Estudar a influência da suplementação da vitamina D no dano ao DNA de células do hipocampo de animais com lúpus induzido por pristane.

5.2 Objetivos específicos

- Padronizar a técnica do ensaio cometa em tecido cerebral congelado;
- Analisar danos ao DNA de células do hipocampo em modelo experimental de lúpus através do ensaio cometa alcalino;
- Analisar a histopatologia do hipocampo para avaliação da área do hipocampo;
- Analisar o infiltrado de IgG na região do hipocampo;
- Analisar a expressão do Receptor de Vitamina D (VDR) na região do hipocampo.

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ARTIGO 1

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Pristane-Induced Lupus as a potential experimental model of Neuropsychiatric Lupus?

Thaís Evelyn Karnopp[1,2,3]; Eduarda Correa Freitas[1,3]; Gustavo Flores Chapacais[1,3,4]
Odirlei André Monticielo[1,2,3]

[1] Laboratório de Doenças Autoimunes, Divisão de Reumatologia, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil.

[2] Programa de Pós-Graduação em Medicina: Ciências Médicas, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

[3] Serviço de Reumatologia, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

[4] Curso de Graduação em Biomedicina, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Correspondence: Rua Ramiro Barcelos, 2350, Centro de Pesquisas Experimentais, sala 12109, Porto Alegre 90035-003, Brasil

Corresponding author: Thaís Evelyn Karnopp, tekarnopp@gmail.com
Telephone number: +55 51 3359 8837

Abstract

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease characterized by an abnormal response to self-antigens that can affect many organs and tissues. SLE patients may have neurological complications such as depression, seizures, headache and mood disorders, which characterize neuropsychiatric lupus (NPSLE). Research in humans is difficult because of the great heterogeneity of the disease. Animal models are a resource for new discoveries, both for disease pathologies and for new treatments. In this review, we approach lupus experimental models that present neuropsychiatric manifestations. Spontaneous animal models such as NZB/W F1 and MRL/*lpr* are commonly used in NPSLE researches; these models present few SLE symptoms when compared to the induced animal models, such as uplemen-induced lupus (PIL). PIL model is known to present eight of the main clinical and laboratory manifestations of SLE, described by the American College of Rheumatology. Many cytokines associate with NPSLE are express in PIL model, like IL-6, TNF-α and IFN. However, to date, NPSLE manifestations have been poorly studied in the PIL model. In this review article, we hypothesize and discuss whether the PIL model is capable of mimic neuropsychiatric manifestations of SLE.

Keywords: Systemic Lupus Erythematosus; Neuropsychiatric Lupus; Lupus Animal Models; Pristane-Induced Lupus, Neuropsychiatric Lupus Animal Model

1. Introduction

Interesting reports on systemic lupus erythematosus (SLE) have been discussed, although remain many gaps in delineating the exact physiopathology mechanisms contributing to the development of the disease [1–3]. SLE is a chronic autoimmune disease characterized by the overproduction of antinuclear autoantibodies (ANA) resulting in the formation of immune complexes (IC) that induce tissue inflammation and destruction in multiple organs and tissues [4,5]. The nervous system may be affected and this is associated with a worse prognosis and more accumulative damage in the patients [6,7].

Neuropsychiatric systemic lupus erythematosus (NPSLE) pathogenesis has not yet been well characterized, but it is believed that an important factor is B cell-mediated damage and physiological, pathological or traumatic actions that can lead to a disruption of the blood-brain barrier (BBB). Thus, immune system cells and autoantibodies can cross this barrier by promoting an inflammatory environment with glial activation, neurodegeneration, and consequent adverse behavioral outcomes [8,9].

Human research is difficult because of the heterogeneity of the disease. NPSLE generates numerous manifestations encompassing both the central and peripheral nervous system, with symptoms that range from thrombotic events to disorders affecting cognition, mood, and level of consciousness [10]. Also, it is difficult to perform a brain tissue biopsy in humans, with imaging exams being an alternative to study. Histological analysis after death could be done, but there are also difficulties in performing because of rapid tissue decomposition of the brain. Therefore, animal models are a resource for new discoveries, both for dissect the cellular and genetic mechanisms enveloped NPSLE, as well as to identify therapeutic targets and screen treatments [11,12]. In this review, we approach the main findings of experimental models that present NPSLE manifestations and hypothesize whether the PIL model is able of mimic neuropsychiatric manifestations of described in the literature to the present moment.

2. Systemic Lupus Erythematosus (SLE)

SLE is a chronic autoimmune disease characterized by an abnormal response to self-antigens that can affect many organs and tissues. It is

considered an unpredictable, recurrent and remittent disease, as well a heterogeneous disease [13]. SLE is one of the most prevalent autoimmune disorders with a striking 9:1 female to male bias, affecting women between childbearing age and menopause [14]. This difference is still misunderstood, being recently associated with the female hormones that may be related to activation of the immune system, contributing to some manifestations of the disease [15–19].

Clinical heterogeneity often makes diagnosis and management challenging even for experienced physicians, including mucocutaneous manifestations, arthritis, serositis, hematologic disorders, glomerulonephritis, neuropsychiatric alterations and others [19]. The pathogenesis of SLE hinges on loss of tolerance and sustained autoantibody production, including abnormalities in apoptotic cell clearance, altered cytokine profiles, and dendritic cell, neutrophilic extracellular traps, B and T cell activation [19–23]. SLE is highly associated with an imbalance between apoptotic cell production and the disposal of apoptotic material [20,21]. This defect allows the presentation of the autoantigen and the beginning of the autoimmunity cascade [24,25].

Apoptotic debris is usually clean quickly and would not be accessible to the immune system. However, in SLE the excessive accumulation of apoptotic debris, containing mainly nuclear material, can stimulate antigen-presenting cells like dendritic cells and B cells [20,25]. These cellular interactions lead to the generation of antinuclear antibodies through interactions with autoreactive T cells [21,26]. T cells activate B cell responses and infiltrate target tissues causing damage. Several cytokines are involved in SLE autoimmune priming and the tissue injury inflammatory mediated, as for example interferon I (IFN-I), IFN-II, tumor necrosis factor (TNF), B lymphocyte stimulator (BLys), interleukin 6 (IL-6), IL-17, IL-18 [27,28].

Usually, macrophages are involved in the phagocytosis of dead cells and the release of cytokines also take part in the process, with the increased secretion of anti-inflammatory cytokines (transforming growth factor beta ,TGF-β and IL-10) and the decreased secretion of the proinflammatory cytokines (such as IL-12 and TNF-α) [29,30]. Since this mechanism inefficiency happens to be associated with lack of tolerance to autoantigens, an impairment in the clearance of cell debris by macrophages has been observed in patients with

SLE [29,30], but why this whole system is harmed in lupus patients is yet to be more clarified.

Another mechanism worth mentioning is NETosis, a type of cell death involving specifically neutrophils, which occurs considerably faster than the usual apoptosis, extruding nuclear material – known as neutrophil extracellular traps (NETs), usually used against pathogens. Since many pro-inflammatory cytokines are involved in inducing NETosis and the levels of NET proteins are found higher in SLE patients than in health individuals, there is an indication that these molecules participate in inflammatory situations [31].

3. Neuropsychiatric lupus (NPSLE)

SLE patients may have neurological complications when the nervous system is affected. Symptoms may develop suddenly or in periods of remission and recurrence over time, and the location and extent of the lesion may vary [16]. These neurological complications are known as NPSLE, affecting more than 50% of SLE patients [32,33]. The NPSLE manifestations encompassing both the central nervous system (CNS) and peripheral nervous system (PNS). Indeed, peripheral symptoms are present in 2-3% of cases [34]; combined paresthesia, pain, autonomic dysfunction, peripheral ataxia, weakness, and atrophy are some common manifestations [35]. On the other hand, CNS complications can be more frequent and classified in focal or diffuse [36]. Diffuse manifestations are responsible for 75% of CNS events and that are often transient, reversible on therapy, and not associated with brain pathologic abnormalities [33,37,38].

Neuropsychiatric impairment in an individual depends on genetic, environmental, and hormonal factors. Among the several symptoms presented in this condition, the most frequent are a headache, mood disorders, cognitive dysfunctions, seizures and cerebrovascular disease [6]. The pathogenesis of NPSLE is poorly understood. Vascular abnormalities, autoantibodies and inflammatory mediators are hypothesized as primary contributory factors [39]. As well, some studies have shown the role of BBB breakdown [40–43] and neuronal damage [44–47] as a trigger on NPSLE development.

3.1 The Blood–Brain Barrier Dysfunction

In general, the blood-brain barrier (BBB) forms an interface between the blood and the brain that protect the brain from circulating autoantibodies, unwanted compounds, and cells [48]. The BBB is composed primarily of endothelial cells, astrocyte end-feet, pericytes, perivascular macrophages, and a basal membrane. This barrier is the result of the tightly sealed monolayer of endothelial cells with tight junctions and adherens junctions. The BBB allows the free diffusion of certain small essential water-soluble nutrients, while other complex nutrients rely on highly selective transport systems to enter the brain [49].

Alteration of the BBB forming cells and/or disruption of the tight junctions can cause increased permeability, resulting in the influx of proinflammatory molecules, cells, and autoantibodies that upset normal brain function and lead to neuronal injury [42]. It has been demonstrated that excessive amounts of neurotransmitters, cytokines, chemokines and peripheral hormones may influence the permeability of the BBB [48].

Jafri et al. [50] described a model of injury in NPSLE, developed in 3 stages. Initially, the periphery produces antineuronal antibodies. Soon after, there is a breach of BBB, which may or may not be related to SLE, followed by neuronal damage mediated by antibodies. In that sense, BBB integrity can be hampered by sepsis, bacterial infections, trauma, cerebral ischemia as well as stress [51]. Studies with rodents observed that the rupture of BBB occurs in a regional way, according to the injury. These studies demonstrate that, after bacterial stimulation of lipopolysaccharides, there is an influx of IgG only in the hippocampus and that the systemic administration of adrenaline allows the passage of IgG into the amygdala [52–54].

Recently was shown that not only the BBB disruption can lead to neuropsychiatric manifestations, also, the cerebral spinal fluid (CSF) dysfunction can activate the triggers for NPSLE [55]. The authors infer that the blood-CSF barrier dysfunction facilitates the exposition of the brain to antibodies and abnormal lymphocytes entry on cerebral ventricles. At least twenty autoantibodies in serum and CSF have been related in NPSLE and they could cross the barrier when your integrity is compromised [52,56–58].

Also, a mutation on *Trex1* gene can lead to disruption on BBB. This gene is associated to type 1 IFN-α mediated autoinflammation due to the deposition of intracellular nucleic acid [59,60]. Studies have shown a severe CNS involvement when polymorphisms of this gene were present [61,62]. Besides IFN-α, a variety of cytokines have been identified as inflammatory mediators, these cytokines have a pathogenic role and can lead to BBB dysfunction, such as TNF-α, IL-1, IL-6, IFN-γ, and TNF–like weak inducer of apoptosis (TWEAK) [32,41,63,64].

Autoantibodies also can lead to BBB dysfunction, anti-N-methyl-D-aspartate receptor antibodies (anti-NMDAR) and anti-ribosomal-P antibodies are circulating neuropathic antibodies and not really access the brain, but have been report as effectors in NPSLE [65,66]. Therefore, it can be inferred that the effect of a potential antineuronal antibody depends on the location of BBB infection, and this depends on the injury induced thereby altering the variation of the manifestations observed in the NPSLE [50].

Cellular infiltrates have been found in both humans and animal models. A T cell infiltrate, especially in the choroid plexus stroma, has been reported in the MRL/*lpr* model [67]. However, B cells play an important role in the presentation of antigens to T cells, facilitating their activation. B cells secrete various cytokines, such TNF and IL-6, contributing to local inflammation and mediated leukocyte infiltration [68,69].

4. Animal models of NPSLE

Animal models are an excellent resource for new discoveries of the diseases. For SLE there are many types of animal models, being spontaneous or induced models. The problem is that due to SLE being a heterogeneous disease, none of these models are capable to reproduce the entire clinical spectrum found on these disease [1,70].

Most animal models for lupus produce autoantibodies, immune complexes and develop glomerulonephritis. The models vary from spontaneously developing animals, such as MRL/*lpr* and NZB/W F1 model, and induced animal models, such as pristane-induced lupus (Balb/c e C57BL/6J) [71,72]. However, although we have several models of lupus well described in the literature, we have few animal models for NPSLE described. The NPSLE models most commonly used are MRL/*lpr* and NZB/W F1 mice models. The

clinical, molecular and neuropsychiatric manifestations of NZB/W F1, MRL/*lpr* and pristine-induced lupus models are shown in table 1.

Due to frequent complications in the CNS, such as behavioral and cognitive dysfunction associated with SLE and difficulty in accessing the amplitude of the manifestations, investigations have been carried out with animal models in the last years, looking to better understand the pathophysiology of the disease [12,73].

5. Spontaneous models of NPSLE

5.1 *NZB/W F1*

NZB/F1 is the oldest classic model of lupus. In 1960s, a cross between New Zealand black (NZB) and New Zealand white (NZW) strains gave rise to strain NZB/W F1 [74]. Both NZB and NZW present limited manifestations of autoimmunity, while NZB/W F1 developed severe autoimmune disease with autoantibodies and defect on clearance of immune complexes [75]. They also present several manifestations of lupus-like disease, including the predominance in female, splenomegaly, elevated serum of ANA mostly directed against DNA (anti-dsDNA), production of cytokines IL-6, TNF-α, IFN-γ, immune complex-mediated nephritis, leading to renal failure and death [76,77].

This strain develops neuropsychiatric manifestations including learning and memory deficits, which develop late during the course of the disease [78]. Also, shown to have mononuclear infiltration in cerebral and hippocampal blood vessels and in the choroid plexus. It presents neurological deficits such as reduction in levels of different neuropeptides in the cortex, hippocampus and hypothalamus [79]. The disadvantages of this model are the late development and slow progression of the disease [80].

5.2 MRL/ lpr

A spontaneous mutation in the autosomal recessive gene *lpr* causes a defect on the expression of the *Fas* gene, characterizing the Medical Research Laboratories - MRL/*lpr* strain. This mutation is responsible for alteration in transcription of Fas receptor, resulting in lymphadenopathy, elevated levels of T cells, high autoantibodies titers, and failure of lymphocytes to undergo to

apoptosis [81–84]. This model develops a fast and aggressive lupus-like disease characterized by immune-mediated damage to kidney, skin, heart, lungs, joints, brain. As well, it produces autoantibodies against dsDNA and Smith antigen (Sm) [85].

Increased serum levels of cytokines IFN-γ, IL-10, C-X-C motif chemokine 10 (CXCL10) and C-C Motif Chemokine Ligand 2 (CCL2) accompany the early development of neuropsychiatric symptoms [86]. Also, high levels of IgG in the CSF and changes in brain structure are demonstrated in mice with neuropsychiatric impairment [87,88]. The complement role in NPSLE pathogenesis has been explored in both human and mouse models [89,90]. A research using MRL/*lpr* mouse found an association between complement infiltrate and BBB disruption [91].

Some researches with MRL/*lpr* mice showed a tendency of these animals to present neuropsychiatric symptoms, such as cognitive dysfunctions and anxiety. To confirm the pathological condition, some tests are needed, such as anxiety, depressive behavior and disease-like, cognitive and motor [84]. Anxiety can be assessed through tests such as elevated-plus maze and open field. In general, when compared to healthy mice, MRL/*lpr* mice show an increase in anxiety levels by decreased exploration [92]. The forced swim test is a test to access the depressive behavior of these animals, the MRL/*lpr* mice show increased floating time and despair behavior, which indicate a performance deficit and a depressive behavior [93].

In addition to the tests performed with the animals, it is possible to perform post-mortem examinations, such as IgG markers, albumin dosing, inflammatory cytokines and cellular infiltrates in the brain [84,88,92].

Cytokine production in SLE appears to be associated with the disease phenotype. Have been identified as inflammatory mediators and may disrupt the BBB, per example IL-6 is highly expressed in patients with CNS manifestations [63,94]. They play an important role in cognitive functions, such as learning and memory [95]. The unregulated production of proinflammatory cytokines may be associated with neuropsychiatric manifestations in SLE, such as memory deficit and emotional disorders, and are already associated with depression in humans [96–99].

The MRL-*lpr* model express increased serum levels of proinflammatory cytokines (TNF-α, IL-1β and IL-6) [100–102]. Also, proinflammatory cytokines genes for IFN-γ, IL-1, IL-6 and IL-10 are overregulated in the hippocampus of MRL/*lpr* mice, these cytokines are associated with cognitive and emotional dysfunctions in SLE [103].

5.3 Others models

There are others animal models useful for determine some NPSLE pathways to disease development. One of these is the 564Igi strain, which has a slow disease progress and an invariant IgG knock-in with RNA autoreactivity leading to the development of a lupus-like phenotype [104–106].

Another model is the B6, which carries the same MRL/*lpr Fas* mutation. This model manifests behavioral deficits and increase neuronal death. Nevertheless, this phenotype is probably attenuated compared to MRL/*lpr* [107]. Recently was developed a new strain, B6.Sle1/Sle3 (Sle1,3) is a bicongenic mice, derived from the introgression of 2 lupus susceptibility loci from the NZM2410 spontaneous SLE model on C57BL/6 (B6) mice. These knockout mice present a significant increase in brain infiltrating leukocytes, cognitive and affective behavioral deficits [108].

6. PIL as a neuropsychiatric model?

Pristane-induced lupus (PIL) is a lupus model using C57BL/6J and BALB/c strains for induction, the latter being the most used. Known to be the most successful model to mimic the human manifestations of the disease, when compared to spontaneous models [3].

The disease induction is done through an intraperitoneal injection with 0.5 ml of pristane, where the oily solution causes an immune response [3]. This model is characterized by the production of specific autoantibodies of SLE, proteinuria, serositis, increased expression of TNF-α, arthritis, and glomerulonephritis [109–111]. Inflammatory cytokine production plays an important role in PIL. The stimuli of autoantibodies formation are made by IL-6, IL-12, IFN-I, IFN-γ on this model [112,113]. These cytokines promote the production of autoantibodies as anti-dsDNA, anti-ssDNA, anti-ribossomal P, anti-Sm and anti-Su [109,114–117].

Studies show that increased expression of TNF and its receptor (TNFR1) induces inflammation and degradation of the CNS [118]. Was demonstrated that both MRL/*lpr* and PIL models present an increase in TNF levels in serum [101,111]. Also, the PIL model express increased IFN-I and IFN-γ and a strong IFN signature, characteristic of the disease in patients [111,119,120]. In contrast, NZB/W mice have poor expression of the IFN signature and are absent in MRL/*lpr* mice [121]. While IFN-I is detectable in two weeks after pristine induction, autoantibodies only appear after three months of the disease induction [122].

It is known that proinflammatory cytokines, such IL-6 and IL-1 can induce cognitive, emotional and spatial learning experimental impairment. MRL/*lpr* mice up-regulate these genes on hippocampus, also express increased levels in serum [123–127]. Animals with PIL have an increased expression of IL-6 in serum [111,120]. The model present hypergammaglobulinemia and production of ANA, similar to the human SLE [128].

Related to NPSLE, Jeltsch-David and Muller [129], point out that there are no descriptions of neuropsychiatric manifestations in animals with induced lupus. However, one study showed that BALB/c mice with PIL tend to decrease their spontaneous locomotion activity [130], similar to what occurs in the MRL/*lpr* model as previously mentioned, this decrease in their locomotion activity may be associated with depression, as in the MRL/*lpr* model and in lupus patients [92].

Recently was describe a downregulation of hippocampal NR2A subunits of NMDA, which mediate certain forms of synaptic plasticity and learning, related to cognitive impairment in PIL model. The authors demonstrate that the pristane could have a potential role in disrupt the BBB by producing anti-Sm antibodies. They also found learning and memory disturbance and associated to downregulation of NR2A [131]. PIL is a very recent model, described in 1994 by Satoh [109], compared to MRL/*lpr* described first in 1978 by Murphy and Roths [132], beginning his discoveries about lupus in the 1980s [133]. Publications about PIL model have been increasing every year and consequently our knowledge about it. It still has unexplored gaps, especially about the neuropsychiatric manifestations in the model.

Table 1. Characteristics of animal models and their neuropsychiatric manifestation

	NZB/NZW F1	**MRL/*lpr***	**PIL**
Type of animal model	Spontaneous	Spontaneous	Induced
Major autoimmune manifestations	- Splenomegaly; - Severe glomerulonephritis; - Hemolytic anemia; - Pulmonary infiltrates	- Severe glomerulonephritis; - Heart infarcts; - Pneumonitis; - Arthritis; - Skin disease	- Proteinuria; - Serositis; - Arthritis; - Glomerulonephritis; - Pulmonary capillaritis; - Anemia;
NP manifestations	- Learning and memory deficits;	- Early depression; - Anhedonia; - Apathy; - Anxiety; - Decreased locomotion; - Impaired spatial learning;	Learning and memory deficits; ????
Molecular and biochemistry manisfestation	- Production of cytokines IL-6, IFN-γ, TNF-α; - Mononuclear infiltration on brain; - Elevated serum ANA; anti-dsDNA; - Week interferon signature.	- Elevated levels of CD4, CD8,CD3, IFNγ, IL-10, IL-6, IL-1β; - Autoantibodies: anti-dsDNA, anti-Sm; high levels of IgG;	- Cytokines expression: TNF-α, IFN-α IFN-β, IFN-γ, IL-6, IL-12; - Autoantibodies: anti-dsDNA, anti-ssDNA, anti-Sm/RNP and anti-ribosomal P, ANA; - Hypergammaglobulinemia; - Strong interferon signature;

ANA: antinuclear antibody; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor;

7. Conclusion

PIL is the animal model that best mimics SLE symptoms. It has recently been shown that there is a break in the BBB in the model, probably due to the production of anti-Sm antibodies. Also, a learning and memory deficiency was described in the model. Compared with well-established NPSLE models, such as NZB/W F1 and MRL/*lpr*, PIL has many similar manifestations to those presented by such models and yet, there is the manifestation of diverse other symptoms. Therefore, we believe that, with further investigations and focused on neuropsychiatric symptoms, the animal model PIL is a possible model of NPSLE induced, with several manifestations further increasing its similarity with human disease. Studies with the PIL model with focus on neuropsychiatric manifestations are necessary to increase the knowledge about this.

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Disclosure statement

The authors declare that they have no competing interests.

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ARTIGO 2

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IgG infiltrate increases Vitamin D Receptor in the hippocampus in pristane-induced lupus model

Thaís Evelyn Karnopp[1,2,3]; Eduarda Correa Freitas[1,3], Alexandre Rieger[4], Gustavo Flores Chapacais[1,3], Odirlei André Monticielo[1,3]

[1] Laboratory of Autoimmune Diseases, Division of Rheumatology, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

[2] Post-graduation Program in Medicine: Medical Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

[3] Division of Rheumatology, Department of Internal Medicine, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

[4] Graduate Program in Health Promotion, University of Santa Cruz do Sul - UNISC, Santa Cruz do Sul, RS, Brazil.

Correspondence: Rua Ramiro Barcelos, 2350, Centro de Pesquisas Experimentais, sala 12109, Porto Alegre 90035-003, Brasil

Corresponding author: Thaís Evelyn Karnopp, tekarnopp@gmail.com
Telephone number: +55 51 3359 8837

Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease that affects many organs and tissues including the central nervous system, a condition known as neuropsychiatric lupus (NPSLE). When the blood-brain barrier breaks down, there is an influx of immune cells, proinflammatory cytokines and autoantibodies. The treatment with vitamin D aims to alleviate symptoms, since it has a promising effect in some studies and also a neuroprotective effect. The animal model of pristane-induced lupus (PIL) mimics some of the SLE symptoms in humans. Despite presenting several symptoms, the model is poorly studied regarding the neuropsychiatric manifestations. In this sense, the objective was to evaluate the influence of vitamin D supplementation on the DNA damage on hippocampus cells, as well as VDR expression, IgG infiltrate and hippocampus size in a PIL model. Twenty-three female BALB/c mice were divided into 3 groups, controls (CO; n= 7), pristane-induced lupus (PIL; n= 9) and pristane-induced lupus supplemented with vitamin D (VD; n= 7). An alkaline comet assay was performed to detect DNA damage in hippocampal cells, measuring hippocampal size, VDR expression, and IgG infiltrate in the hippocampus. The PIL group had a higher infiltrate of hippocampal IgG when compared to the CO group (p= 0.01). Vitamin D has shown a potential to reduce the IgG infiltrate. The VD group presented numerically greater DNA damage when compared to the PIL and CO group. There was no increase in VDR expression in the treated group. The hippocampus size resembled between the groups. There was a positive correlation between the expression of VDR and the IgG infiltration (r= 0.7585, p= 0.0001) in the hippocampus. Our data suggest that the PIL model has the potential to present neuropsychiatric manifestations, since there was an increase in IgG infiltration. Increased infiltration of IgG into the hippocampus indicates an inflammatory process that may have stimulated the increase of VDR expression in induced animals as an attempt to decrease the inflammatory process caused by the disease. To prove this mechanism more effectively, further studies with the PIL model are needed to assess the neuropsychiatric manifestations in mice.

Keywords: Comet Assay, Brain, NPSLE, Vitamin D, DNA damage.

1. Background

Systemic lupus erythematosus (SLE) is an autoimmune inflammatory disease, characterized by a chronic inflammation and autoantibodies production, that can affect any organ and system, such as the central nervous system (CNS) [1–5]. Women are more affected by the disease, with a ratio of 9:1 compared to men. Worldwide incidence rates range from 0.3–23.7 per 100,000 person-years and the disease prevalence is 6.5 to 178.0 per 100,000 person-years [6,7]. In SLE there is an excessive production of autoantibodies and pro-inflammatory cytokines. An environment with many autoantibodies, cytokines, chemokines and peripheral hormones could lead to an increased permeability of the blood-brain barrier (BBB) and even its dysfunction [8]. When it happens, there is an influx of immune cells, autoantibodies and cytokines, causing an inflammatory environment and neurodegeneration [9]. These immunological changes could cause several symptoms such as headache, seizures, cognitive dysfunction, mood disorders and cerebrovascular disease. This condition has been known as Neuropsychiatric Lupus (NPSLE) [10].

The inflammation resulting from the periods of exacerbation generates an uncontrolled increase in free radicals generation, which exceeds the antioxidant capacity of the organism, leading to an oxidative stress environment. This stress causes damage to proteins, lipids and DNA structures and contributes to chronic inflammation [11–14]. New therapies to prevent or even decrease these immune changes have been investigated and vitamin D emerges as a promising alternative in this context.

In the immune system, vitamin D promotes inhibition of B cell proliferation and differentiation, decreasing immunoglobulin secretion [15,16]. Also, it suppresses T cell proliferation, inhibits inflammatory cytokines production (IL-17 and IL-21) and increases anti-inflammatory cytokines production (IL-10) [17–19]. Vitamin D plays an important role in promoting neuron survival. In addition, vitamin D could suppress oxidative pathways in the brain by decreasing oxygen free radical production [20]. Vitamin D receptor (VDR) has been detected in some brain regions, like hippocampus, suggesting that vitamin D may have a neuroprotective properties [17,21,22].

Experimental lupus models can be a good alternative to best study immunological mechanisms that develop NPSLE. Pristane-induced lupus (PIL) is an animal model characterized by the production of specific autoantibodies to SLE, IFN signature, proteinuria, serositis, and glomerulonephritis [23]. In addition, there is reactive oxygen species (ROS) production and DNA damage [24]. Recently, it has been demonstrated that pristane may have a potential role in BBB disruption in BALB/c mice [25]. However, there are no studies demonstrating the implications of DNA damage in NPSLE, as well as the effect of vitamin D administration in this context. Therefore, this study aims to evaluate the influence of vitamin D supplementation on the DNA damage on hippocampus cells in a PIL model.

2. Materials and Methods

2.1 Ethics

This study was approved by the HCPA Animal Ethics Committee (number 18-0246) and used biological samples from a previous study (number 17-0011). Moreover, it was conducted in accordance with the National Institute of Health guidelines.

2.2 Animals and Experimental Design

Twenty-three female BALB/c mice were randomly divided into the following three groups: 1- Control group (CO; n=7); 2- Pristane-induced lupus group (PIL; n=9); 3- Pristane-induced lupus treated for six months with vitamin D group (VD; n=7). The CO group received a single intraperitoneal (i.p.) injection with 500 µl 0,9% saline solution and the PIL and VD groups received a single i.p. injection with 500 µl pristane oil (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich, MO, USA), according to Satoh et al. [26]. During the procedures, mice were anaesthetized with isoflurane 10% (Abbott Laboratório do Brasil Ltda.,Brazil) and 90% of oxygen.

The VD group mice received a subcutaneous injection of Calcijex (Abbott Labs, Chicago, Ill) containing 2 µg/kg/day of calcitriol (1,25-[OH]2-D3) in PBS-Tween 20 buffer [15,27], according to Freitas et al. [28]. Subsequently, the injections were also given every second day until the end of the

experiment. CO and PIL groups received subcutaneous injections with PBS-Tween 20 buffer on the same days. Vitamin D supplementation has been initiated since the day of disease induction. Six months after pristane induction, animals were killed and tissue and blood was collected.

2.3 Biological samples

Blood sample was collected by cardiac puncture at the end of the experiment (180 days), prior to euthanasia of the animals. Blood was obtained in vacutainer tubes (BD Biosciences, San Diego, CA); serum was prepared within 45 minutes of collection and stored at -80º C for later analysis.

The brains were dissected into right and left hemispheres. The right hemisphere of the brain was immersed in 10% buffered formalin for fixation for 24h at room temperature, after embedded in paraffin (used entirely for histological analysis). The left hemisphere was separated in regions and was fixed in phosphate-buffered saline (PBS) plus 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, MO, USA) frozen at -80°C [29] and the hippocampus was stored in a separated falcon. All samples were stored in solution with a total volume of 20x the sample volume. Isolated hippocampus was used for comet assay analysis. Firstly, to isolate the hippocampus, the frontal cortex and cerebellum were removed, thus access to the hippocampus was facilitated and removed from the remaining parts of the brain and immediately submerged in PMS 10% DMSO solution.

2.4 Comet Assay

The samples were thawed for a maximum period of three weeks and processed by the comet assay (CA) technique. The CA alkaline was performed according to Singh et al. [30] with adaptations according to Hu et al. [29].

A total of 100 cells were visually scored by measuring the DNA migration length and the amount of DNA in the “tail” into five classes, ranging from undamaged (class 0) to maximally damaged (class 4) [31]. In this way, the damage frequency (DF), the percentage of damaged cells, and the damage index (ID) was calculated for each sample, ranging from 0 (no damage, 100 cells x0) to 400 (maximum damage, 100 cells x4).

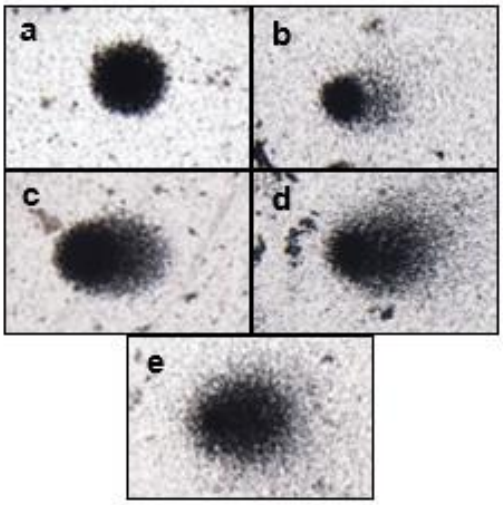

Figure 1. Classes of DNA damage in mice hippocampal cells. The pictures were taken by the authors; a: class 0; b: class 1; c: class 2; d: class 3; e: class 4. Authors, adapted from Collins (31).

2.5 Histology

Sections of tissues were stained with hematoxylin-eosin (HE) and analyzed under optical microscopy (Olympus, Germany) by the blinded pathologist and hippocampal size was measured by ImageJ software (National Institute of Health, Bethesda, MA, United States). The pathologist analyzed B and T cell infiltration and neuronal viability.

2.6 Detection of VDR in hippocampus by immunohistochemistry

Vitamin D Receptor (VDR) in brain was examined by immunohistochemistry. Brain was cut and its sections were submit to dewaxing and stained for VDR with rabbit polyclonal antibody to Vitamin D Receptor – ChIP Grade (1:500 dilution; Abcam – ab3508, USA). All sections were incubated with 10 mM pH 6.0 citrate buffer for 35 minutes and heated in a water bath at 94ºC for 10 min. To block endogenous peroxidase activity sections were immersed in a 5% H_2O_2 in methanol solution for 20 min. To block nonspecific antigen binding, sections were pre-incubated for 20 min with 5% skim milk with PBS. VDR antibody was suspended in PBS and 0.1% BSA (bovine serum albumin), incubated at 4ºC overnight. Secondary antibodies Goat anti-rabbit IgG, Peroxidase Conjugated (1:200 dilution, Millipore -

AP132P) was incubated at room temperature for 1 hour and 30 minutes. Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB) chromogen solution, and the slides then counterstained with Mayer's hematoxylin and mounted. The images were captured in a microscope Olympus BX51 (Olympus, Germany) with Olympus camera. Five photos at a final magnification of 400x, were captured per animal in the hippocampus region between zone C1 and dentate gyrus. Color intensity was digitized and quantified by ImageJ software.

2.7 Detection of IgG in hippocampus by immunofluorescence

IgG infiltrate in hippocampus was examined by direct immunofluorescence. To stained for IgG with goat polyclonal antibody anti-mouse IgG-FITC (1:50 dilution; Abcam – ab97022, USA), paraffin blocks containing the left-brain hemisphere were cut in section. The sections were submitted to dewaxing in hothouse at 75ºC for 40 minutes. After, the sections were immersed in three xylols for 5 minutes each, then in four 99% alcohols and washed in distilled water. For antigen recovery, all sections were incubated with 10 mM pH 6.0 citrate buffer for 35 minutes and heated in a water bath at 94ºC for 10 minutes. For protein blocking the sections were incubated with 3% BSA in 0.05% PBS-Tween 20 for 1 hour. All antibodies were incubated with PBS-Tween 20 containing 2% BSA in a humid incubator for at 4ºC overnight. Sections were washed three times for 5 minutes using PBS-Tween 20 and 50µl DAPI was added to each tissue section, after 20 minutes the slide was mounted. Images were captured in microscopy confocal Leica TCS SP8 (Leica Microsystems, HE, Germany) at a final magnification of 400x. Five photos were captured per animal in the hippocampus region between zone C1 and dentate gyrus. Fluorescence intensity was scanned and quantified by LAS X (Leica Microsystems, HE, Germany) software.

2.8 Statistical Analysis

The results were inserted in the specific program of statistical analysis Statistical Package for the Social Sciences (SPSS), version 18.0. The data will be presented as mean and standard deviation or standard error median (minimum-maximum), DI was quantify in arbitrary units (a.u.) and expressed in

percentage. Kruskal-Wallis test and Spearman correlation was used. GraphPad Prism 6 was used for the graphics. For all the tests, a risk of 5% ($p \leq 0.05$) and its respective confidence interval of 95% were assumed.

1. Results

The alkaline comet assay was performed after euthanasia and no difference was observed between CO and PIL groups. Animals treated with vitamin D visually showed (figure 2) greater DNA damage than CO and PIL group. Although there is no statistically significant difference between the VD group compared to PIL and CO groups, an increased damage could be observed in this group.

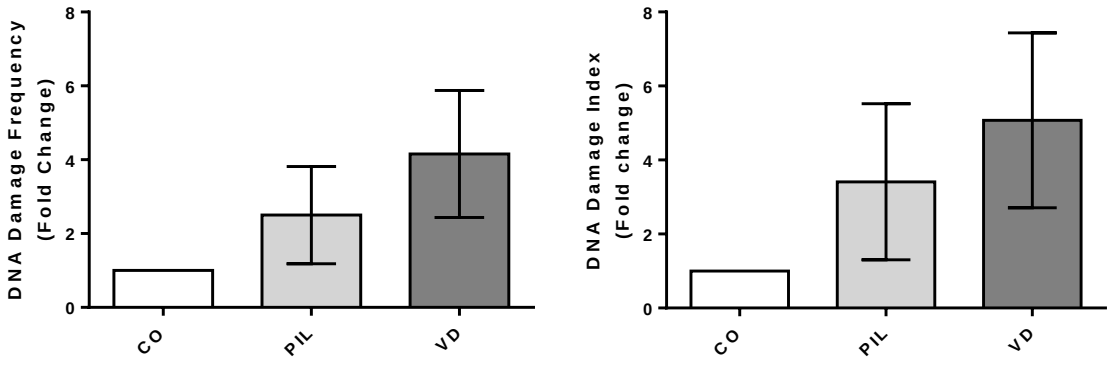

Figure 2. DNA damage index and damage frequency in hippocampal cells in mice with pristine-induced lupus. Data are presented as fold change relative to CO group. Control group mean values for hippocampus DNA damage index were set at value of 1. One animal of CO group was excluded for mechanic damage. Values are expressed as mean±SEM. $p<0.05$; Statistical analysis *p*: p value, significance value $p \leq 0.05$, Kruskal-Wallis test. CO: controls; PIL: Pristane-induced lupus; VD: Pristane-induced lupus with vitamin D.

The size of hippocampus was measured in histological sections stained with HE. There was no difference between the CO and PIL groups (Figure 3) regarding hippocampal size. It was also not possible to observe a change in hippocampal size between the PIL and VD groups. Furthermore, it was not possible to detect B-cell infiltrates and neuronal viability in the HE slides.

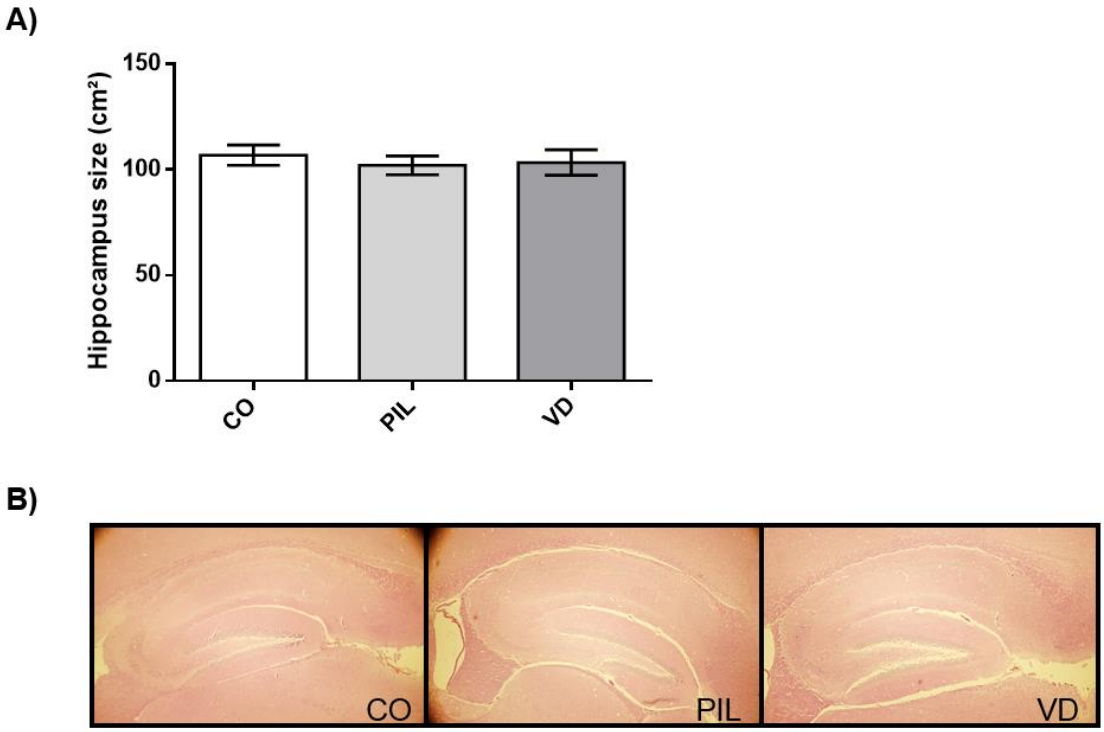

Figure 3. Morphology and hippocampus size of BALB/c mice in HE. A) Values are expressed as mean±SEM. Statistical analysis *p*: p value, significance value ≤0.05, Kruskal-Wallis test. B) Hippocampal morphology between groups CO, PIL and VD. Images was observed under microscopy (x200) and analyzed and measured by ImageJ. CO: Controls; PIL: Pristane-induced lupus; VD: Pristane-induced lupus with vitamin D.

In immunohistochemistry, it was possible to visualize the expression of VDR in the hippocampus region, especially in the PIL group, although there was no difference between the CO and PIL groups. Regarding the treatment, VD group showed no increase in protein expression compared to the PIL group (figure 4).

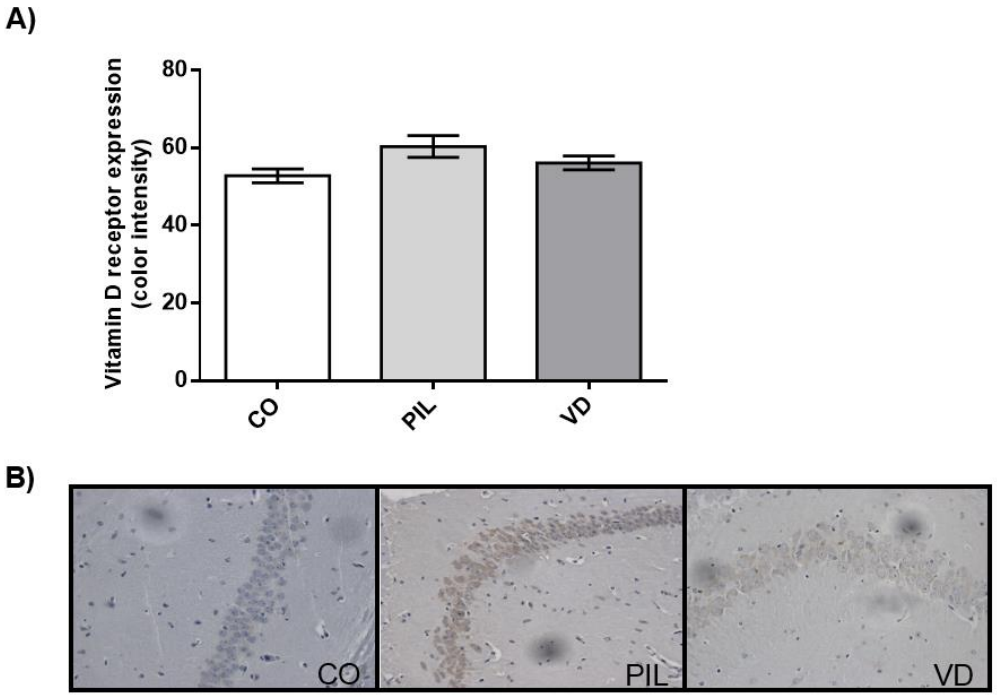

Figure 4. Expression of Vitamin D Receptor (VDR) in hippocampus zone in pristine-induced lupus. A) Values are expressed as mean±SEM. Statistical analysis *p*: p value, significance value p≤0.05, Kruskal-Wallis test. B) VDR expression (color intensity) in hippocampus of BALB/c mice in each group was observed under microscopy (x400) and analyzed and calculated by ImageJ. CO: Controls; PIL: Pristane-induced lupus; VD: Pristane-induced lupus with vitamin D.

To access total IgG deposit in the hippocampus, an immunofluorescence assay was performed. Compared to CO animals, the PIL group significantly increased total IgG infiltrate in the hippocampus (*p*= 0.01). Additionally, vitamin D showed a potential to reduce the hippocampal IgG infiltrate (table 1 and figure 5).

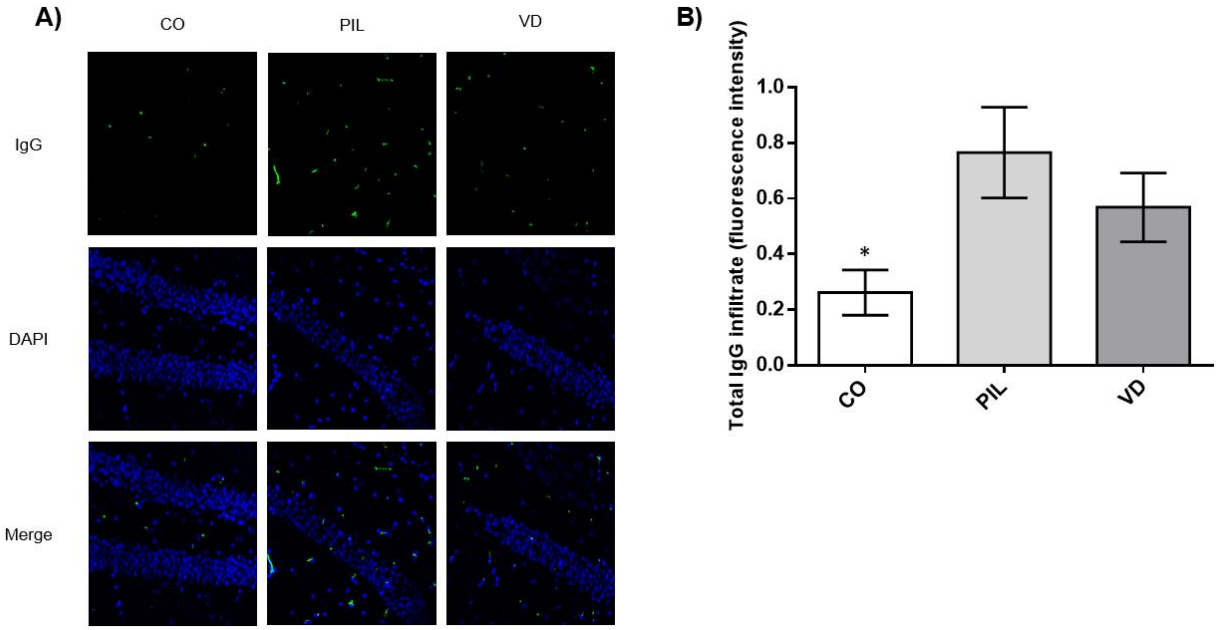

Figure 5. Total IgG deposit in hippocampus zone in pristine-induced lupus. A) Fluorescence intensity of total IgG in hippocampus of BALB/c mice in each group was observed under fluorescence confocal microscopy (x400) and analyzed and calculated by LAS (X); B) Difference between fluorescence intensity of total IgG on CA1 to CA4 hippocampus region. **p*=0.01. Values are expressed as mean±SEM. Statistical analysis *p*: p value, significance value ≤0.05, Kruskal-Wallis test. One CO animal was excluded due to technical error in immunofluorescence. CO: Controls; PIL: Pristane-induced lupus; VD: Pristane-induced lupus with vitamin D.

Table 1. Expression of Vitamin D Receptor and total IgG infiltrate in hippocampus zone of BALB/c mice.

	CO (n= 7)	PIL (n= 9)	VD (n= 7)	*p*
VDR	55.08±7.37	60.28±8.33	56.09±4.70	0.26
IgG	0.26±0.19	0.76±0.49	0.56±0.32	0.01

Values are express as mean±SD; VDR: Vitamin D Receptor; IgG: Immunoglobulin G; Statistical analysis: One CO animal was excluded due to technical error in immunofluorescence. *p*: p value, significance value ≤0.05, Kruskal-Wallis test. CO: Controls; PIL: Pristane-induced lupus; VD: Pristane-induced lupus with vitamin D.

For a better investigation of the relationship between VDR and total IgG infiltrate in the hippocampus, we performed the Spearman correlation test. The results show that there is a positive correlation between VDR and IgG infiltrate (figure 6). In both PIL and VD groups the correlation are stronger (PIL r= 0.7726 and VD r= 0.7864) then in CO group (r= 0.0675).

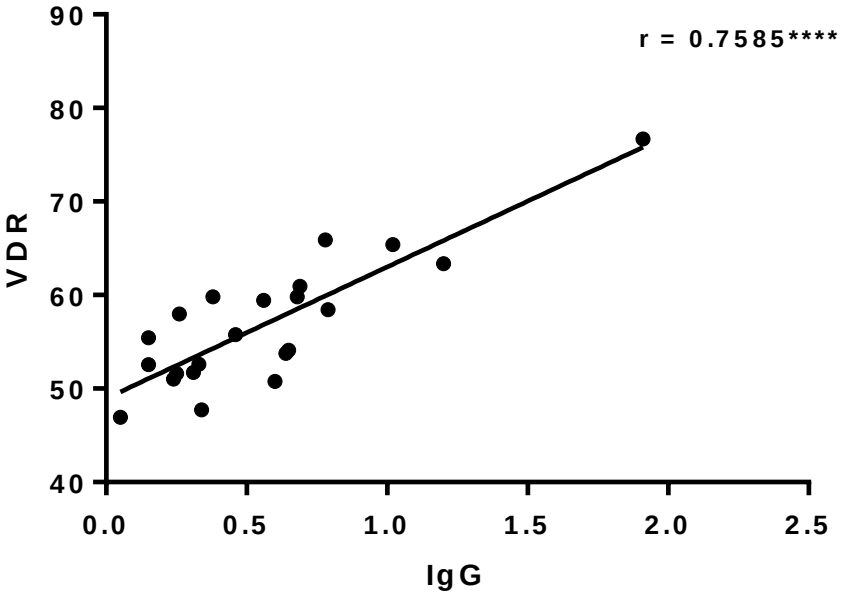

Figure 6. Correlation between VDR expression and total IgG infiltrate in hippocampus of BALB/c mice. *p=0.0001 with r= 0.7585. Values are express as mean±SD; Statistical analysis p: p value, significance value ≤0.05, Spearman correlation test. One CO animal was excluded due to technique error in immunofluorescence. CO: Controls; PIL: Pristane-induced lupus; VD: Pristane-induced lupus with vitamin D.

2. Discussion

SLE affects many organs and systems, including the CNS [5,32]. Inflammation during periods of disease exacerbation increases the levels of ROS and it could be detrimental to DNA [33,34]. Vitamin D is able to cross the BBB naturally and may play a neuroprotective role [35–37]. At the moment, there is one study demonstrating neuropsychiatric manifestations in PIL model [25]. To the best of our knowledge, there are no studies associated with DNA damage of hippocampal cells and lupus model, as well as the implications of vitamin D treatment in mice with PIL. However, in this study, our findings have not been able to demonstrate high DNA damage in animals with PIL, nor a reduction in DNA damage in animals treated with vitamin D. We believe that these results are due to the small sample size of our study. It is also possible that these cells were able to adapt to the chronic inflammatory stress. The alkaline comet assay evaluates an acute injury that may not have been detected by the technique, since the analysis was performed three weeks after tissue collection and not in fresh tissue.

Studies have associated hippocampal atrophy with NPSLE. We evaluated the hippocampal size of the animals and found no differences between groups. Contrary to our results, MRL/*lpr* mice demonstrated, through magnetic resonance imaging (MRI), a decrease in size in several areas of the brain, like limbic and midbrain structures and a retarded growth of the hippocampus resulting in atrophy of the region [38]. As we did not evaluate animals over time with MRI, only at the final time of 180 days, it was not possible to compare hippocampal development according to the disease course. Moreover, MRL/*lpr* mice that manifest cognitive impairment showed improvement in symptoms after supplementation with vitamin D [39]. These animals were treated at the same dose as in our study (2 µg/kg/day), but were treated daily for 4 weeks, while we treated each two days for a period of 6 months.

Vitamin D supplementation has numerous benefits such as the regulation of calcium absorption and immunomodulation [40]. Our results did not show differences between the VDR in hippocampus of VD animals compared to PIL or CO. Vitamin D dose and frequency of treatment influences VDR expression and could lead to high dose toxicity, with severe kidney and heart complications. High doses of vitamin D may exceed the capacity of the catabolic and elimination pathways, leading to the accumulation of one or more vitamin metabolites. Thus, VDR can be activated irregularly and there may be a decrease in receptor expression at some tissues [41]. From this, it can be inferred that the treatment used may have been an interfering factor in the final results.

Once there is a disruption of the BBB, there is an influx of immune cells, autoantibodies and cytokines that did not cross the barrier naturally. The hippocampus is one of the most affected regions, with memory and cognition deficits. In this context, we quantified total IgG present in the hippocampus zone. There is a greater influx of IgG on hippocampus of animals with PIL when compared to CO. Treatment with vitamin D, demonstrated a tendency to reduce the influx of these antibodies. Similarly, MRL/*lpr* animals showed an increase in total IgG in serum and cerebrospinal fluid, and were not statistically different from controls [42].

Correlation data showed us that the higher the expression of VDR, the greater the amount of IgG infiltrate. The CO group showed a weak correlation, which indicates low levels of IgG in the hippocampus. *In vitro* studies revealed that the addition of vitamin D in B cell culture leads to apoptosis. When treatment is initiated early in the culture, it suppresses the production of immunoglobulins such as IgG [43,44]. It can be inferred that VDR expression in both PIL and VD animals increases with the amount of IgG as an attempt to suppress the production of this antibody, corroborating with the total hippocampal infiltrate IgG data.

The present results highlights the amplitude of signs and symptoms presented in the PIL model and the potential that this model has to be studied as an NPSLE model. Recent evidence demonstrated the potential of this model to be the newest induced NPSLE model [25]. MRL/*lpr* and NZB/W are well-established models of NPSLE, but they are spontaneous models that do not show many symptoms of SLE when compared to induced models such as PIL. In this context we seek to expand the knowledge about BALB/c mice with PIL.

As this is a pilot study, some limitations, such as the small sample size, brain collection not made by formaldehyde perfusion and the samples stored in paraffin may have influenced the negative results. We performed a comet assay on frozen tissue as described by Hu [29] instead of fresh tissue and it could be a limitation of our study. For further clarification it is necessary to conduct a new study with comet assay in fresh tissue. To have more information about the implications of the PIL model in neuropsychiatric manifestations, it is necessary to continue the study with a new methodology with a longer experimental time.

1. Conclusions

This is the first study to evaluate DNA damage in hippocampal cells in PIL, as well as to analyze the total IgG infiltrates and VDR expression in the hippocampus of these animals. Our data suggest that the PIL model has the potential to present neuropsychiatric manifestations, since there was an increase in IgG infiltrate on hippocampus. To prove more effectively, further studies with the PIL model are needed in order to assess neuropsychiatric

manifestations in mice. In addition, it has been shown that vitamin D supplementation may play a neuroprotective role but in our study vitamin D was not able to reduce DNA damage of hippocampal cells. However, increased infiltration of IgG into the hippocampus may have stimulated the increased VDR expression in induced animals as an attempt to decrease the inflammatory process caused by the disease. Finally, the results suggest that vitamin D may play a different role depending on the pathways in the body, especially on the brain.

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Conflict of interest

The authors declare that they have no competing interests.

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8. CONSIDERAÇÕES FINAIS

Neste estudo foi padronizada a técnica de ensaio cometa com células congeladas, descrito em detalhes na metodologia expandida (anexo 1). Também foi avaliada a influência da suplementação de vitamina D na neuroproteção em modelo experimental de lúpus induzido por pristane. Demonstrou-se que a vitamina D não foi capaz de reduzir os danos causados ao DNA, através do uso da técnica de ensaio cometa. O tamanho do hipocampo não se alterou entre os grupos.

Os animais do grupo PIL apresentaram um maior infiltrado de IgG total no hipocampo, quando comparados aos animais CO. Estes resultados demonstram uma possível quebra da BHE e um potencial deste modelo ter induzido NPSLE. Apesar da suplementação com vitamina D não ter reduzido significativamente os níveis de infiltrados de IgG total na região do hipocampo, foi possível observar uma tendência da vitamina D em diminuir esses infiltrados, atuando de forma neuroprotetora. Entretanto, para se confirmar tal achado, serão necessários novos estudos com um maior número amostral.

A suplementação também não alterou a expressão de VDR no hipocampo. Em contrapartida, foi possível correlacionar a expressão de VDR com o infiltrado de IgG. Os resultados demonstraram que o aumento da expressão de VDR pode estar associada com o aumento de infiltrado de IgG, talvez em uma tentativa de diminuir o processo inflamatório na região.

8. PERSPECTIVAS FUTURAS

Este estudo foi pioneiro em relação a avaliação de danos ao DNA de células do hipocampo em modelo murino de LES e a demonstrar infiltrados de IgG total no hipocampo destes animais.

Na continuidade deste trabalho, pretendemos investigar mais a fundo as implicações do NPSLE no modelo de lúpus induzido por pristane, explorando com as manifestações clínicas, bem como as alterações morfológicas, comportamentais moleculares e celulares no cérebro destes animais.

9. CONSIDERAÇÕES GERAIS

A presente dissertação de mestrado é fruto do trabalho realizado no Laboratório de Doenças Autoimunes no Centro de Pesquisa Experimental vinculado ao Serviço de Reumatologia do Hospital de Clínicas de Porto Alegre. A participação de professores, médicos contratados, veterinários, outros funcionários contratados, alunos de mestrado, doutorado e bolsistas de iniciação científica foi fundamental para a idealização e realização desta pesquisa.

Durante o vínculo como aluna de mestrado do Programa de Pós-Graduação em Medicina: Ciências Médicas da UFRGS, que iniciou em janeiro de 2018, a autora participou das seguintes produções científicas:

1. Freitas EC, Karnopp TE, Silva JMS, Santo RCE, Rosa TH, Oliveira MS, Gonçalves FC, Oliveira FH, Schaefer PG & Monticielo OA. Vitamin D supplementation ameliorates arthritis but does not alleviates renal injury in pristine-induced lupus model. Autoimmunity. 2019 (52)2: 69-77.

2. Publicação de resumo no Annual Book do EULAR 2019. Freitas EC, Karnopp TE, Silva JMS, Santo RCE, Rosa TH, Oliveira MS, Gonçalves F, Oliveira FH, Schaefer PG, Monticielo O. 1,25 dihydroxyvitamin d improvements arthritis but does not alleviate renal damage in pristane-induced lupus model. Annals of the Rheumatic Diseases, volume 78, supplement 2, year 2019, page A1542.

3. Apresentação de pôster na 39ª Semana científica do HCPA. Trabalho intitulado “avaliação de dano ao DNA do hipocampo de camundongos com lúpus induzido por pristane suplementados com vitamina D”. Autores: Chapacais GF, Karnopp TE, Freitas EC, Rieger A, Monticielo OA.

4. Apresentação oral de trabalho no SIC UFRGS com premiação de destaque. Trabalho apresentado pelo bolsista voluntário Gustavo Flores Chapacais, intitulado “Dano ao hipocampo no lúpus eritematoso sistêmico: avaliação em camundongos com lúpus induzido por pristane e suplementados com vitamina D”.

5. Chamada Universal CNPq, nº 28/2018, contemplados com R$ 29.996,00.

6. Apoio da Sociedade de Reumatologia do Rio Grande do Sul, com valor de R$ 21.000,00.

10. ANEXOS

Anexo 1 – Metodologia Expandida

10.1 Delineamento Experimental

Trata-se de um projeto experimental comparativo, onde foram utilizadas amostras de encéfalos de camundongos, pertencentes a um projeto já executado, armazenadas em blocos de parafina (hemisfério direito) e fixadas em PBS + 10%DMSO congeladas a -80Cº (hemisfério esquerdo).

10.1.1 Animais

Para diminuir o número de animais utilizados pelo grupo de pesquisa, os tecidos utilizados neste projeto foram coletados de animais incluídos em um projeto de doutorado aprovado no CEUA – HCPA sob número 17-0011.

No referido projeto, foram utilizados 28 camundongos BALB/c fêmeas de 8-12 semanas de idade. Os animais foram mantidos em gaiolas com água e comida *ad libitum* no biotério climatizado da Unidade de Experimentação Animal do Hospital de Clínicas de Porto Alegre com temperatura, umidade, fluxo de ar e ciclo de luz controlado (12/12-claro/escuro).

10.1.2 Modelo Experimental LES Induzido por Pristane

O modelo experimental de LES foi induzido por pristane em 20 camundongos fêmeas BALB/c através de uma única injeção intraperitoneal (IP) de 500µl de pristane (2,6,10,14 tetramethylpentadecane; Sigma- Aldrich) previamente filtrado em membrana 0,22 µm. Os animais controle receberam a mesma quantidade de solução salina 0,9% pela mesma via (figura 5) (107).

Grupos experimentais

- Grupo 1: camundongos Balb/C controles saudáveis (n=8);

- Grupo 2: camundongos Balb/C com LES induzido por pristane (PIL) (n=10);

- Grupo 3: camundongos Balb/C com LES induzido por pristane (PIL) + uplementação de 1,25-dihidroxivitamina D (n=10).

10.1.3 Tratamento

Todas as aplicações de suplementação foram realizadas no período da manhã, entre as 8:00-10:00 horas, com o intuito de evitar qualquer alteração circadiana. Os camundongos foram alocados aleatoriamente em conjuntos de 10 animais por grupo e foram tratados por via subcutânea com solução salina 0,9% ou 1,25 dihidroxivitamina D (Calcijex, Aboot labs) na concentração de 2ug/kg diluído em PBS-Tween 20 (108) em dias alternados, com solução final de 100ul (figura 5). O tratamento foi realizado por 180 dias, iniciando após a indução da doença.

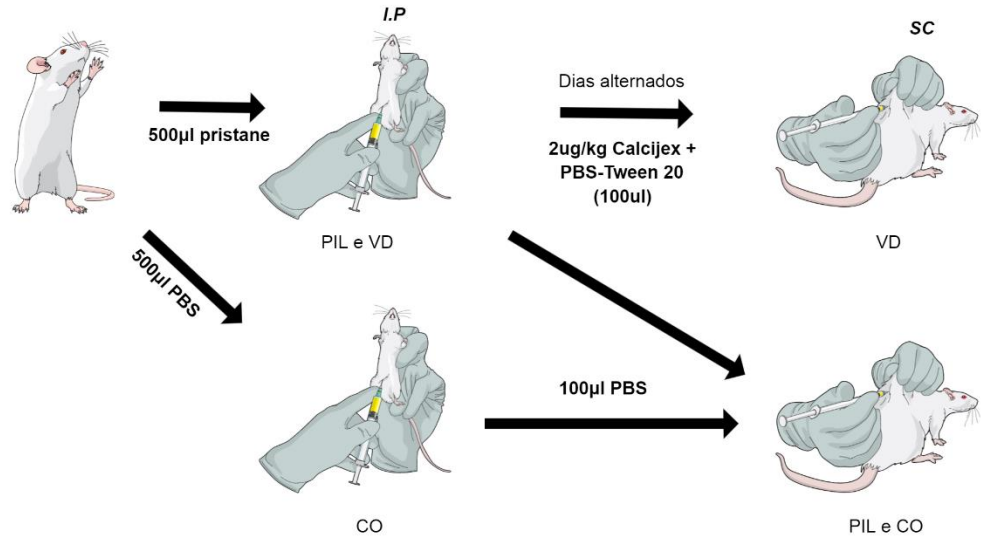

Figura 5. Esquematização da indução de lúpus induzido por pristane e tratamento. Os animais dos grupos PIL (lúpus induzido por pristane) e VD (lúpus induzido por pristane suplementados com vitamina D) receberam uma injeção intraperitoneal contendo 500µL de pristane para indução da doença. Enquanto que o grupo CO (controle) recebeu uma injeção de PBS, pela mesma via, passando assim pelos mesmos procedimentos que os demais grupos. O tratamento com vitamina D (Clacijex – concentração de 2ug/kg + PBS-Tween 20, totalizando 100ul de solução) foi realizado por via subcutânea a cada dois dias no grupo VD. O grupo PIL e CO receberam 100ul de PBS pela mesma via, nos mesmos dias. Fonte: Autor.

10.2 Tecido

Após a coleta de sangue, os animais receberam uma sobredose anestésica de isofluorano por via inalatória, até promoção de parada cardiorrespiratória. Somente após a confirmação da morte, foi retirado o encéfalo que foi dividido entre hemisfério cerebral direito e hemisfério cerebral esquerdo. Sendo o hemisfério direito acondicionado em Formol tamponado (10%) e armazenado em blocos de parafina após 24 horas, para posterior análise histológica.

Para o hemisfério esquerdo a região do hipocampo foi separada. Primeiramente, para isolar o hipocampo, o córtex frontal e o cerebelo foram removidos, facilitando o acesso ao hipocampo, retirando-o das partes remanescentes do cérebro (figura 6) e imediatamente acondicionado em tubos tipo falcon contendo 20mL de PBS 1x e 10% de Dimetil Sulfóxido (DMSO), que foi congelado a -80ºC para posterior análise através do ensaio cometa, o mesmo processo de congelamento foi feito para as demais regiões do encéfalo (109).

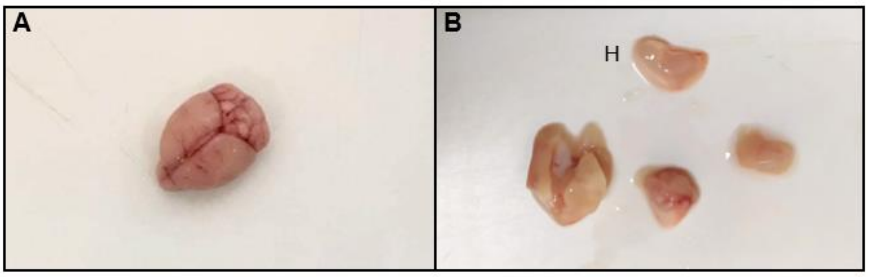

Figura 6. Encéfalo de camundongo BALB/c. A: Encéfalo inteiro logo após a retirada do crânio; B: Partes do hemisfério esquerdo; H: Hipocampo. Fonte: Autor.

10.3 Ensaio Cometa (EC)

Para avaliação dos danos ao DNA foi realizado o Ensaio Cometa versão alcalina, o ensaio foi realizados no Laboratório de Doenças Autoimunes do Hospital de Clínicas de Porto Alegre. As etapas do EC, descritas a seguir, seguem o protocolo descrito por Singh *et al.* (110) com adaptações realizadas conforme Hu e colaboradores (109).

10.3.1 Confecção das Lâminas e Lise Celular

Foram pipetados 20µL de suspensão celular e misturados em 80µL de Agarose de baixo ponto de fusão (0,75%). A ressuspensão foi colocada em uma lâmina de microscopia previamente pré coberta com agarose (1%). Imediatamente, as lâminas foram cobertas com lamínulas (24x50 mm) e colocadas em um refrigerador (cerca de 4°C), por 5 minutos para que a solução (agarose e células) ganhasse consistência, diminuindo assim os riscos de descolamento da superfície durante as etapas subsequentes. Após esse procedimento, as lamínulas foram retiradas e as lâminas colocadas em cubeta vertical envolta em papel alumínio contendo Solução de Lise (2.5M NaCl, 100mM EDTA, 10Mm Tris, pH 10 com 1% de Triton X-100 e 10% de DMSO) refrigerada, para remoção de membranas e proteínas celulares. As lâminas foram mantidas nestas condições por 24 horas.

10.3.2 Eletroforese Alcalina

Após 24 horas, as lâminas foram retiradas da Solução de Lise e submetidas a um Tampão Alcalino de eletroforese (300mM NaOH, 1mM EDTA, pH 13), por 15 minutos, para permitir o desenovelamento do DNA. Posteriormente, foi aplicado uma corrente elétrica de 25 V e 300 mA por 20 minutos para que ocorresse a migração dos fragmentos de DNA.

10.3.3 Neutralização e Fixação

Após a eletroforese, as lâminas foram submersas em Solução de Neutralização (0.4M Tris, pH 7) durante 5 minutos por 3 vezes. Depois de secas foram fixadas com Solução Fixadora (15% TCA, 5% ZnSO4, 5% Glicerol).

10.3.4 Coloração e Contagem das Lâminas

A coloração foi realizada com nitrato de prata, como descrito por Nadin; Roig; Ciocca (111) e analisadas em microscopia óptica convencional. Foram analisadas 100 células por amostra (50 de cada lâmina em duplicata), sendo essas classificadas visualmente em cinco classes, de acordo com o tamanho da cauda: sem danos (classe 0) até dano máximo (classe 4) (Figura 7) (103).

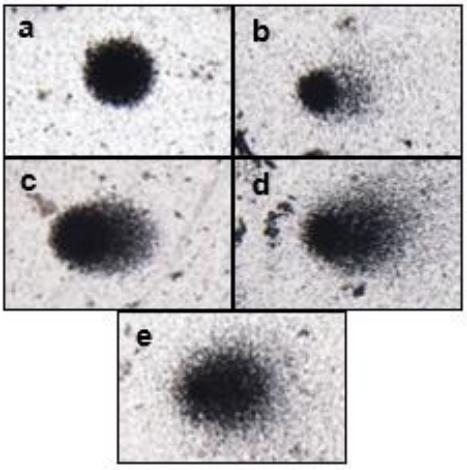

Figura 7. Fotografia ilustrativa, mostrando a classificação dos níveis de dano no DNA (0-4) em células do hipocampo de camundongos BALB/c. Níveis de danos (a) dano 0; b) dano 1; c) dano 2; d) dano 4) detectados pelo ensaio cometa, onde 0 é sem dano e 4 é o maior nível de dano encontrado. E) Células que podem estar entrando em apoptose. Fonte: Autor.

Dessa forma, foram calculados a frequência de dano (DF), a porcentagem de células danificadas, e o índice de dano (ID), que variam de 0 (sem dano, 100 células x 0) a 400 (máximo de dano, 100 células x 4) por indivíduo.

10.4 Análise Histopatológica do Encéfalo

Seções de tecidos foram coradas com hematoxilina-eosina (HE) e analisadas sob microscopia óptica (Olympus, Alemanha) por um patologista cegado e o tamanho do hipocampo foi medido pelo software ImageJ (Instituto Nacional de Saúde, Bethesda, MA, Estados Unidos). O patologista analisou a infiltração de células B e T e a viabilidade neuronal.

10.5 Imunohistoquímica – VDR

A expressão do receptor de vitamina D (VDR) no cérebro foi examinada por imuno-histoquímica. Cortes longitudinais de 3μm de espessura foram obtidos no micrótomo na Unidade de Patologia Experimental do CPE do HCPA para a realização da reação de imunohistoquímica, que foi realizada pelo método da estreptavidina-biotina-peroxidase. As seções foram submetidas à desparafinização e coradas com anticorpo anti-VDR policlonal

de coelho – Grau ChIP (diluição 1: 500; Abcam – ab3508, EUA). Todas as seções foram incubadas com tampão citrato 10mM pH 6,0 por 35 min e aquecidas em banho-maria a 94ºC por 10 min. Para bloquear as atividades endógenas da peroxidase, as seções foram imersas em uma solução a 5% de H_2O_2 em metanol por 20 minutos. Para bloquear a ligação não específica ao antígeno, as seções foram pré-incubadas por 20 min com leite em pó desnatado a 5% com PBS. O anticorpo VDR foi suspenso em PBS e BSA (albumina de soro bovino) a 0,1%, incubado a 4ºC *overnight*. Após, foram realizadas lavagens com PBS para retirada do anticorpo primário e então as seções foram incubadas com anticorpo secundário IgG de cabra, anti-coelho, conjugado com peroxidase (diluição 1: 200, Millipore – AP132P) à temperatura ambiente por 1 hora e 30 minutos. A reação imunológica foi visualizada usando a solução de cromogênio 3,3'-diaminobenzidina (DAB), e as lâminas foram contracoradas com hematoxilina de Mayer e montadas. As imagens foram capturadas no microscópio de captura Olympus BX51 (Olympus, Alemanha) com câmera Olympus. Cinco fotos com uma ampliação final de 400x foram capturadas por animal na região do hipocampo entre a zona C1 e o giro dentado. A intensidade da cor foi digitalizada e quantificada pelo software ImageJ.

10.6 Imunofluorescência – IgG total

O infiltrado de IgG total no hipocampo foi examinado por imunofluorescência direta. Para coloração foi utilizado o anticorpo policlonal de cabra anti-IgG-FITC (diluição 1:50; Abcam – ab97022, EUA). Os blocos de parafina contendo o hemisfério esquerdo do cérebro foram cortados em seções com 3µm de espessura. As seções foram submetidas à desparafinização em estufa a 75ºC por 40 minutos. Após, as seções foram imersas em três xilóis por 5 minutos cada, depois em quatro álcoois a 99% e lavadas em água destilada. Para recuperação do antígeno, todas as seções foram incubadas com tampão citrato 10mM pH 6,0 por 35 min e aquecidas em banho-maria a 94ºC por 10 min. Para o bloqueio de proteínas, as seções foram incubadas com BSA a 3% em PBS-Tween 20 a 0,05% por 1 hora. Todos os anticorpos foram incubados com PBS-Tween 20 contendo BSA a 2% em uma incubadora úmida por 4ºC *overnight*. As seções foram lavadas

três vezes por 5 min usando PBS-Tween 20 e 50µl de DAPI foram adicionados a cada seção de tecido, após 20 minutos a lâmina foi montada. As imagens foram capturadas em microscopia confocal Leica TCS SP8 (Leica Microsystems, HE, Alemanha) com uma ampliação final de 400x. Cinco fotos foram capturadas por animal na região do hipocampo entre a zona C1 e o giro dentado. A intensidade da fluorescência foi digitalizada e quantificada pelo software LAS X (Leica Microsystems, HE, Alemanha).

10.7 Análise estatística

Os resultados foram inseridos em um programa específico de análise estatística *Statistical Package for the Social Sciences* (SPSS), versão 18.0. Os dados serão apresentados em média e desvio padrão ou erro padrão, mediana (mínimo-máximo), o ID foi quantificado em unidades arbitrárias (u.a.) e expresso em percentual. Foi utilizado o teste de Kruskal-Wallis e a correlação de Spearman. O GraphPad Prism 6 foi usado para os gráficos. Para todos os testes, assumiu-se um risco de 5% ($p \leq 0,05$) e seu respectivo intervalo de confiança de 95%.

Anexo 2 – Consort 2010 Guideline

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1[a]	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	7
Introduction			
Background and objectives	2[a]	Scientific background and explanation of rationale	17
	2b	Specific objectives or hypotheses	35
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	73
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	73
Participants	4a	Eligibility criteria for participants	73
	4b	Settings and locations where the data were collected	73
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	73
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	73
	6b	Any changes to trial outcomes after the trial commenced, with reasons	N/A
Sample size	7a	How sample size was determined	73
	7b	When applicable, explanation of any interim analyses and stopping guidelines	N/A
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	N/A
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	N/A
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	N/A
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	N/A
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	N/A
	11b	If relevant, description of the similarity of interventions	73
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	73
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	73
Results			
Participant flow (a	13a	For each group, the numbers of participants who were randomly	77

diagram is strongly recommended)		assigned, received intended treatment, and were analysed for the primary outcome	
	13b	For each group, losses and exclusions after randomisation, together with reasons	77
Recruitment	14a	Dates defining the periods of recruitment and follow-up	N/A
	14b	Why the trial ended or was stopped	N/A
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	N/A
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	N/A
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	N/A
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	N/A
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	N/A
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	N/A
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	82
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	82
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	82
Other information			
Registration	23	Registration number and name of trial registry	N/A
Protocol	24	Where the full trial protocol can be accessed, if available	N/A
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.