UNIVERSIDADE PAULISTA – UNIP

ESTUDO DOS EFEITOS MORFOLÓGICOS E COMPORTAMENTAIS DA ADMINISTRAÇÃO DE DOXORRUBICINA ASSOCIADA À CURCUMINA E AO RESVERATROL EM RATOS WISTAR

Dissertação apresentada ao Programa de Pós-Graduação em Patologia Ambiental e Experimental da Universidade Paulista - UNIP, para a obtenção do título de Mestre em Patologia Ambiental e Experimental.

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DEDICATÓRIAS

Dedico esta dissertação aos meus pais Antônio Carlos Moretti e Neide Larocca Moretti, que foram fundamentais para a minha formação; a minha irmã Fernanda Larocca Moretti, pelo carinho e estímulo; aos meus amigos que estiveram ao meu lado, me apoiando e dando força nestes dois anos de dedicação.

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RESUMO

A doxorrubicina (DOX) é conhecida por causar prejuízos cognitivos em pacientes submetidos a longos períodos de quimioterapia (déficits conhecidos como *chemobrain*). Existe, assim, urgente necessidade por estratégias terapêuticas capazes de permitir o retorno dos sobreviventes de câncer a sua prévia qualidade de vida. A curcumina (CUR), pigmento de coloração amarela extraído de rizomas de cúrcuma (*Curcuma longa L.*), e o resveratrol (RSV), um polifenol natural, possuem atividades antioxidantes e anti-inflamatórias. O objetivo deste estudo foi o de investigar os efeitos comportamentais e morfológicos da administração de CUR e RSV em associação com a DOX, a fim de verificar se tais substâncias podiam aliviar a neurotoxicidade descrita para a DOX. Ratos Wistar, machos adultos, foram divididos em 4 grupos: DOX (2,5 mg/kg/semana por 4 semanas, via intraperitoneal - i.p.), DOX+RSV (DOX, 2,5 mg/kg/semana por 4 semanas, i.p.; RSV, 10 mg/kg/dia por 28 dias, gavagem), DOX+CUR (DOX, 2,5 mg/kg/semana por 4 semanas, i.p.; CUR, 100 mg/kg/dia por 28 dias, gavagem) e controle (CTR, solução salina a 0,9%, i.p.). Foram realizadas análises comportamentais (campo aberto e teste de reconhecimento de novos objetos - NORT). Os encéfalos foram coletados e processados para a análise histopatológica pelas técnicas de coloração *luxol fast blue* e hematoxilina-eosina e imuno-histoquímica para expressão de GFAP (proteína glial fibrilar ácida) em astrócitos e de Iba1 (molécula adaptadora de ligação de cálcio ionizado 1) na micróglia. Os ratos injetados com DOX apresentaram comprometimento da memória de curto e longo prazo, como visto no NORT às 3 e 24 após a habituação, e aumento da expressão de GFAP e de Iba1, respectivamente, em astrócitos e na micróglia do córtex frontal, hipotálamo e hipocampo. Tais déficits cognitivos foram revertidos pela CUR em ambos os períodos e pelo RSV às 24 horas. A astrogliose e a microgliose induzidas pela DOX foram revertidas pelo RSV, assim como pela CUR. Não foram observados sinais de desmielinização e de perda neuronal em qualquer grupo. Dessa forma, a CUR e o RSV se mostraram capazes de reverter a perda de memória, a astrogliose e a microgliose induzidas pela quimioterapia com DOX.

Palavras-chave: astrócitos; déficit cognitivo; micróglia; proteína glial fibrilar ácida; quimioterapia.

ABSTRACT

Doxorubicin (DOX) is known to cause cognitive impairments in patients submitted to long-term chemotherapy (deficits also known as chemobrain). Therefore, there is an urgent need for therapeutic strategies capable of returning cancer survivors back to their previous quality of life. The present study investigated whether resveratrol (RSV) or curcumin (CUR) administration could affect mnemonic function and brain morphological changes following DOX administration in rats. Male Wistar rats were divided into 4 groups: DOX group (2.5 mg/kg/week for 4 weeks, i.p., plus distilled water for 28 days, oral gavage - OG), DOX+RSV group (DOX, 2.5 mg/kg/week for 4 weeks, i.p., plus RSV, 10 mg/kg/day for 28 days, OG), DOX+CUR group (DOX, 2.5 mg/kg/week for 4 weeks, i.p., plus CUR, 100 mg/kg/day for 28 days, OG) and control (CTR) group (0.9% saline solution/week for 4 weeks, i.p., plus distilled water for 28 days, OG). Behavioral analyses (open field - OF - and the novel object recognition test - NORT) were performed. Brains were collected and analyzed by hematoxylin-eosin and luxol fast blue staining techniques and by immunohistochemistry for GFAP (glial fibrillary acidic protein) expression in astrocytes and Iba1 (ionized calcium-binding adaptor molecule 1) expression in microglia. DOX-injected rats presented short-term and long-term memory impairments as seen in the NORT at 3 and 24 hours after habituation and increased GFAP and Iba1 expression, respectively, in astrocytes and microglia of the frontal cortex, hypothalamus and hippocampus. Such cognitive deficits were reverted by CUR at both periods and by RSV at 24 hours. DOX-induced astrogliosis and microgliosis were reverted by RSV and CUR. No signs of demyelination or neuronal loss were found in any group. Thus, CUR and RSV reverted memory loss, astrogliosis and microgliosis induced by DOX monotherapy.

Keywords: astrocytes; chemotherapy; glial fibrillary acidic protein; memory impairment; microglia.

SUMÁRIO

1 INTRODUÇÃO

O comprometimento cognitivo induzido pela quimioterapia, também conhecido como chemobrain ou chemofog, é amplamente reconhecido como um efeito colateral adverso frequente após a administração de drogas quimioterápicas (ASHER; MYERS, 2015; TAILIBERT *et al*., 2016). As deficiências de memória foram amplamente relatadas após protocolos quimioterapêuticos em modelos murinos (KONAT *et al.,* 2008; KITAMURA *et al.,* 2015; RAMALINGAYYA *et al.,* 2016; BARRY *et al*., 2018; CARDOSO *et al*., 2020) e estudos cognitivos em seres humanos (FALLETI *et al.,* 2005, AHLES; SAYKIN, 2007; VARDY; TANNOCK, 2007; ASHER; MYERS, 2015), sendo atribuídas principalmente a danos oxidativos (ALUISE *et al.,* 2010; GAMAN *et al.,* 2016; KEENEY *et al.,* 2018; LIAO *et al.,* 2018; REN *et al.,* 2018) e / ou à neuroinflamação (CHEUNG *et al.,* 2013; TIEN *et al*., 2016; WANG *et al.,* 2015, 2016 ; ORCHARD *et al.,* 2018; SHI *et al*., 2018). Tais alterações podem ser sutis ou dramáticas, temporárias ou permanentes, persistindo por anos e até mesmo podendo piorar com o tempo, assim causando angústia significativa entre os pacientes e impedindo o retorno à qualidade de vida experimentada antes do tratamento (VARDY; TANNOCK, 2007).

A aprendizagem e a memória exigem mecanismos neuronais que permitam mudanças rápidas, mas persistentes nos circuitos cerebrais, constituindo ferramentas importantes para a sobrevivência dos seres vivos, porque representam sua capacidade para adquirir, reter e usar o conhecimento, o que resulta em aprendizagem (SHAPIRO, 2001; EICHENBAUM, 2010).

A base celular da aprendizagem e da memória consiste na remodelagem funcional das conexões sinápticas, sendo frequentemente chamada de plasticidade sináptica, o que inclui tanto a memória explícita ou declarativa, quando a pessoa pode lembrar e descrever algum fato ou evento passado, quanto a memória implícita ou procedural, tal como na habilidade motora aprendida. A memória é frequentemente subdividida em curto prazo (de minutos a horas) e de longo prazo (de dias a toda vida). A formação da memória de curto prazo envolve a modificação das proteínas existentes, frequentemente por

fosforilação. As alterações de longo prazo envolvem a ativação genética, a síntese proteica e o rearranjo da membrana, incluindo a formação e/ou reabsorção dos terminais pré-sinápticos, bem como das espinhas pós-sinápticas. Em alguns estudos, demonstrou-se que o volume de córtex cerebral dedicado a uma tarefa aumenta com treinamento especifico (LANDOWNE, 2011).

O modelo celular de plasticidade neural induzida pela aprendizagem no hipocampo foi denominado de potenciação de longa duração (LTP), definida como um aumento da atividade sináptica após estimulação de alta frequência de uma sinapse química. A LTP tem sido proposta nas últimas décadas como a base para a formação da memória de longo prazo (IZQUIERDO *et al.,* 2008).

A memória sensorial é aquela que nos permite reter as informações que chegam até nós por meio dos sentidos, podendo ser estímulos visuais, auditivos, gustativos, olfativos, táteis ou proprioceptivos. A memória sensorial se caracteriza biologicamente por ser um fenômeno de natureza elétrica. Isso quer dizer que essas informações não produzem alterações morfológicas e nem funcionais nos neurônios envolvidos no processo. Assim, a informação está disponível apenas enquanto os neurônios disparam potenciais elétricos; com o fim desses disparos, perde-se a informação (KIM, 2011).

Estudos de imagem de pacientes após quimioterapia sistêmica mostram que tal tratamento produz alterações estruturais e funcionais no encéfalo, algumas das quais parecem persistir mesmo quando os déficits cognitivos cessaram. Isto sugere que, com o tempo, a plasticidade neural pode ser capaz de compensar os efeitos deletérios do tratamento quimioterápico (HURRIA; SOMLO; AHLES, 2007; BOYKOFF; MOEINI; SUBRAMANIAN, 2009).

Espécies reativas de oxigênio (EROs) e espécies reativas de nitrogênio (ERNs) têm sido associadas a danos nos componentes biomoleculares, incluindo lipídios, proteínas e DNA. Os efeitos deletérios das EROs/ERNs têm sido considerados, fundamentalmente, patogênicos em diversas doenças, como na aterosclerose, no acidente vascular encefálico, na carcinogênese, no envelhecimento e em doenças neurodegenerativas/neuroinflamatórias (GABBITA *et al.,* 2000).

Vários mecanismos foram sugeridos para as alterações estruturais e funcionais no encéfalo após a quimioterapia. Estes incluem alterações inflamatórias centrais e periféricas, desmielinização de tratos da substância branca, redução na proliferação de células estaminais na região neurogênica do hipocampo e de células precursoras de oligodendrócitos (OPCs), assim como modificações em níveis hormonais e/ou de fatores de crescimento (JEAN-PIERRE; McDONALD, 2016). Foram sugeridos possíveis tratamentos, os quais incluem desde intervenções farmacológicas até terapias cognitivo-comportamentais. Alguns desses foram testados apenas em modelos animais, enquanto outros têm produzido vários graus de melhora nas populações de pacientes. Atualmente, não há nenhum tratamento reconhecido como eficaz e uma melhor compreensão das causas do declínio cognitivo experimentado após a quimioterapia se faz fundamental para encontrar maneiras de prevenir ou tratar os efeitos do *chemobrain* (WIGMORE, 2012).

Estudos com animais fortaleceram a hipótese de que a administração de agentes quimioterápicos inicia uma cascata de alterações biológicas, com alterações de curta duração no ambiente das citocinas, induzindo alterações epigenéticas persistentes. Tais mudanças levariam a alterações na expressão gênica, bem como alterações na atividade metabólica e na transmissão neuronal, responsáveis pela geração da experiência subjetiva da cognição (WANG *et al.,* 2015).

Estudos com os encéfalos de animais que tiveram comprometimento cognitivo induzido por doxorrubicina (DOX) apontam a capacidade de este agente quimioterápico aumentar a produção de EROs/ERNs, elevando os níveis periféricos do fator de necrose tumoral alfa (TNF-α), uma citocina pró-inflamatória que atravessa a barreira hematoencefálica (BHE) e é capaz de induzir o estresse oxidativo no parênquima nervoso, afetando negativamente as mitocôndrias e, consequentemente, conduzindo à apoptose neuronal, o que levaria ao comprometimento cognitivo (BUTTERFIELD, 2014).

Astrogliose (BABETS *et al.,* 2016; CARDOSO *et al.*, 2020) e microgliose (ALLEN et al., 2019) foram descritas no encéfalo de animais submetidos à terapia com DOX. Como a droga apresenta uma baixa penetração no encéfalo, foi hipotetizado que sua administração pudesse induzir uma resposta

inflamatória sistêmica, com aumento da passagem de citocinas pró-inflamatórias pela BHE. No encéfalo, tais citocinas poderiam então desencadear a elevação de moléculas oxidativas e de citocinas pró-inflamatórias, que, em conjunto, induziriam a ativação glial (ALLEN *et al.,* 2019; CARDOSO *et al.,* 2020).

Os astrócitos constituem as maiores e mais numerosas células gliais presentes no sistema nervoso central (SNC) dos mamíferos, excedendo o número de neurônios na proporção de 10:1 (BENVENISTE, 1992; HAIM; ROWITCH, 2017). Apesar de sua pronunciada heterogeneidade morfológica e bioquímica, os astrócitos caracterizam-se pela presença de prolongamentos dotados de filamentos intermediários (fibrilas gliais), cujo componente principal é a proteína glial fibrilar ácida (GFAP - *glial fibrillary acidic protein*), servindo como meio de identificação deste tipo celular em estudos *in situ* e em cultivo (MIDDELDORP; HOL; 2011; BRENNER, 2014; YANG; WANG, 2015). Dentre as numerosas funções destas células, destacam-se a manutenção da homeostasia no microambiente neural, exercendo importante papel na detoxificação, na captação de neurotransmissores, especialmente de glutamato e de ácido gama-aminobutírico (GABA), bem como na regulação do pH, da osmolaridade e da concentração iônica do tecido nervoso. Os astrócitos relacionam-se, ainda, com a orientação da migração neuronal durante o desenvolvimento do SNC, com o suporte mecânico para os oligodendrócitos durante a mielinização, com o reparo após agressões no tecido nervoso, com a produção e a secreção de proteínas da matriz extracelular, bem como com a síntese de moléculas de adesão, de fatores neurotróficos e promotores do crescimento de neuritos. Outras funções incluem a indução e a manutenção das características da BHE, a fagocitose de restos celulares e funções imunes, tais como a secreção de citocinas (interleucina 1 - IL-1, IL-3, IL-6, TNF-α e interferon - IFN-α e β) e a expressão de moléculas do complexo principal de histocompatibilidade (MHC) de classe I e II (SOFRONIEW; VINTERS, 2010; OBERHEIM; GOLDMAN; NEDERGAARD, 2012; SCHITINE *et al.,* 2015).

As células microgliais constituem os macrófagos residentes do SNC (KETTENMANN *et al.,* 2011), apresentando-se como células altamente plásticas. Em condições de repouso, a micróglia tem uma morfologia ramificada, caracterizada por corpos celulares pequenos e processos numerosos,

ramificados e longos. A micróglia ramificada escaneia continuamente o microambiente neural em busca de sinais de perigo associados a patógenos ou lesões (RANSOHOFF; PERRY, 2009). Quando um sinal de perigo é detectado, a micróglia sofre rápidas mudanças morfológicas e funcionais, um processo que tem sido chamado de ativação microglial e é caracterizado pelo aumento de tamanho do corpo celular e de seus prolongamentos e pela secreção de citocinas pró-inflamatórias e de espécies reativas (KREUTZBERG, 1996). Eventualmente, as células da micróglia podem se tornar células ameboides capazes de fagocitose (RANSOHOFF; PERRY 2009; OLAH *et al.,* 2011). Embora a ativação microglial forneça uma defesa contra lesões e infecções, a ativação crônica ou excessiva é considerada prejudicial e tem sido implicada em muitos transtornos neurodegenerativos e psiquiátricos (KAUSHIK; BASU 2013).

A molécula adaptadora de ligação de cálcio ionizado (Iba1 - *ionized calcium binding adaptor molecule 1*) é uma proteína de ligação de cálcio de 17 kDa que é expressa constitutivamente e especificamente em todos os tipos de micróglia, sendo amplamente empregada como um marcador imuno-histoquímico para micróglia ramificada e ativada (AHMED *et al.,* 2007; VINET *et al.,* 2012). A ativação microglial está associada ao aumento da expressão Iba1. No entanto, alterações morfológicas de micróglia podem ocorrer sem afetar significativamente a densidade óptica em áreas encefálicas marcadas com Iba1, muito embora a densitometria não forneça nenhuma informação específica sobre a natureza das alterações morfológicas encontradas (HOVENS *et al.,* 2014).

Um estudo recente demonstrou que uma única aplicação de DOX (2,5 mg/Kg, por via intraperitoneal - IP) foi capaz de diminuir em 45% a velocidade de captação do glutamato no córtex frontal e no hipocampo de camundongos, revelando a existência de mudanças na transmissão glutamatérgica em núcleos associados à função cognitiva 24 horas após administração de dose única, sem impacto duradouro na memória espacial (THOMAS *et al.,* 2017).

Independentemente da causa da lesão no SNC, o reparo do tecido é sempre realizado em maior ou menor grau com participação astrocitária. A reação dos astrócitos inclui o aumento de seu número (astrocitose) e de suas dimensões (astrogliose), além de várias outras alterações funcionais, como

espessamento dos feixes de filamentos gliais e consequente aumento da intensidade de marcação de GFAP (EDDLESTON; MUCKE, 1993; MIDDELDORP; HOL; 2011; BRENNER, 2014; YANG; WANG, 2015). Estes fenômenos têm sido referidos como gliose astrocitária, astrocitose e astrogliose reativas, cicatriz glial, ou simplesmente gliose, podendo ser de 2 tipos de acordo com a natureza do dano provocado: isomórfica, na qual os processos astrocitários apresentam-se orientados pelos elementos teciduais preservados e o arranjo dos feixes de filamentos gliais é uniforme e paralelo, e anisomórfica, na qual sua disposição é irregular ao redor de lesão geralmente causadora de dano morfológico grosseiro na estrutura do tecido, com ruptura da BHE (FERNAUD-ESPINOSA; NIETO-SAMPEDRO; BOVOLENTA, 1993; BIGNAMI; DAHL, 1994).

À medida que os astrócitos ficam mais ou menos reativos, é reconhecido que mesmo mudanças sutis na sua expressão molecular apresentam o potencial de exercer pronunciados efeitos sobre as células neurais circundantes (SOFRONIEW, 2009). Existe evidência cumulativa, tanto clínica quanto experimental, de que disfunções astrocitárias e astrogliose têm a capacidade de contribuir ou serem causa primária de muitas desordens do SNC, surgindo, inclusive, o estabelecimento do termo astrocitopatias, originárias da perda das funções benéficas dessas células no microambiente neural e/ou da expressão de seus efeitos detrimentais no tecido nervoso (SOFRONIEW, 2015).

As antraciclinas (ANT) exercem ação por meio da inibição da topoisomerase II (MENNA *et al.,* 2012). As topoisomerases (I e II) são uma importante classe de enzimas responsáveis pela manutenção da topologia do DNA, estando envolvidas no reparo do DNA, na sua transcrição e replicação e na segregação dos cromossomos (WANG, 2002; NITTIS, 2009). Inibição e/ou interferência das funções da topoisomerase levam à morte celular (SINHA; MASON, 2015). A antraciclina doxorrubicina (DOX) é um fármaco quimioterapêutico representante deste grupo, apresentando grande potência e ampla prescrição. As propriedades antineoplásicas da DOX incluem a interferência na replicação de DNA e na síntese de RNA e a formação EROs/ERNs, que levam ao dano oxidativo das membranas celulares (IYVLEVA; IMYANITOV, 2016; OJHA *et al.,* 2016).

A DOX foi descoberta no início da década de 1970 e tornou-se um dos medicamentos mais importantes e amplamente utilizados na terapia anticâncer, sendo eficaz no tratamento de neoplasias sólidas e hematológicas, embora sua administração seja comumente acompanhada de vários efeitos colaterais graves (WEISS, 1992; FOJTU *et al.,* 2017). O mais grave deles é o desenvolvimento de cardiotoxicidade dose-dependente e cumulativa. Ao longo do tempo, muitas estratégias têm sido investigadas para evitar ou, pelo menos, diminuir a disfunção cardíaca induzida pela DOX. Contudo, a atenuação do seu efeito cardiotóxico ainda não é satisfatória (FOJTU *et al.,* 2017).

Estudos recentes realizados *in vitro* apontam para o potencial neurotóxico da DOX, interferindo na atividade sináptica e afetando ambos os mecanismos de transmissão, excitatório e inibitório, no longo prazo (MORUNO-MANCHON *et al.,* 2016a). Em ratos tratados com DOX, foi descrita a capacidade de o quimioterápico induzir a autofagia lisossomal nos neurônios dos animais que receberam a droga, aumentando a formação de vacúolos intracitoplasmáticos, bem como o aparecimento de organelas danificadas, de complexos autofagossomos e de gotículas lipídicas nessas células. É importante ressaltar que foi observado, além do prejuízo à via autofágica-lisossômica, que os animais tratados com DOX também apresentaram acúmulo de lipofuscina, importante pigmento indicativo de dano oxidativo decorrente do envelhecimento tecidual, uma vez que tal composto é comumente observado em animais senis (MORUNO-MANCHON *et al.*, 2016b). Babets *et al.* (2016) observaram que a administração de DOX (1 mg/Kg, semanalmente, durante 4 semanas, via IP) acarretou aumento do conteúdo de GFAP, mensurado por meio da técnica de ELISA, no hipocampo dos animais que receberam o quimioterápico.

A curcumina (CUR) é um pigmento amarelo extraído da *Curcuma longa* um importante componente dos rizomas. Comumente utilizada como tempero e corante alimentício, também tem sido utilizada como cosmético e em preparações medicinais (TOMEH; HADIANAMREI; ZHAO, 2019). As propriedades terapêuticas da CUR têm sido associadas à sua ação antioxidante e anti-inflamatória (GUPTA *et al.,* 2017). O efeito anti-inflamatório da CUR é provavelmente mediado por sua capacidade de inibir as enzimas ciclooxigenase-2 (COX-2), lipoxigenase (LOX) e óxido nítrico sintase induzível (iNOS), enzimas

que medeiam os processos inflamatórios. A regulação inadequada da COX-2 e/ou da iNOS tem sido associada à fisiopatologia de certos tipos de tumores, bem como a distúrbios inflamatórios (ALIBEIKI *et al.,* 2017; TOMEH; HADIANAMREI; ZHAO, 2019). Devido à íntima relação da inflamação com a tumorigênese, prevê-se que a CUR, com sua potente propriedade anti-inflamatória, exerça efeitos quimiopreventivos sobre a carcinogênese (GERA *et al.,* 2017). Assim, as últimas décadas testemunharam numerosas pesquisas dedicadas às propriedades antioxidantes e anti-inflamatórias da CUR.

Estudos clínicos têm demonstrado que a CUR pode ser utilizada como agente neuroprotetor no tratamento da epilepsia, da doença de Alzheimer e de outros distúrbios neurodegenerativos (MENDONÇA *et al.,* 2013). A CUR apresenta ainda ação protetora à neurotoxicidade associada ao haloperidol. Seu uso como pré-tratamento pode prevenir, de forma dose-dependente, as alterações celulares, comportamentais e neuroquímicas induzidas por este fármaco (BISHNOI *et al.*, 2011).

Mendonça *et al.* (2013) observaram que a CUR foi capaz de diminuir a inibição do crescimento de neuritos induzida pela cisplatina em até 50%, além de verificarem a redução da degeneração das fibras nervosas do nervo ciático induzida pelo referido quimioterápico.

A CUR é reconhecida como uma substância segura para humanos e animais, especialmente por administração oral, não sendo mutagênica e, portanto, segura durante gravidez (SOLEIMANI *et al.,* 2018). Estudos em humanos não mostraram efeitos tóxicos e a CUR foi considerada segura na dose de 6 g/dia por via oral durante 4 a 7 semanas, muito embora alguns efeitos adversos, como distúrbios gastrointestinais, possam ocorrer (SOLEIMANI *et al.,* 2018).

O resveratrol (trans-resveratrol, 3,5,4'-tri-hidroxi-trans-estilbeno - RSV) é um polifenol natural não flavonoide, pertencente a uma família de compostos polifenólicos conhecidos como estilbenos e encontrado em alimentos como uvas, vinho, amendoim e soja. Enquanto o amendoim e as uvas contêm baixos níveis de RSV, o vinho tinto fornece concentrações relativamente altas do composto. O RSV é também uma das fitoalexinas, um grupo de substâncias de baixa massa molecular com atividade antimicrobiana, produzidas pelas plantas

como resposta de defesa a alguns estímulos exógenos, como radiação ultravioleta (UV), estressores químicos e, principalmente, infecções microbianas. O composto existe em duas formas isoméricas - o isômero trans ocorre nas peles de baga da maioria das cultivares de uva e sua síntese é estimulada pela luz UV e por infecções fúngicas; o isômero cis é produzido por irradiação UV do transisômero, sendo geralmente ausente ou apenas ligeiramente detectável nas uvas, mas originário da vinificação. A maioria das pesquisas sobre o RSV diz respeito ao isômero trans, devido a sua presença natural nas uvas e a sua maior estabilidade (TRELA; WATERHOUSE, 1996; BAUR; SINCLAIR, 2006; MORENO *et al.,* 2008; SHI *et al*., 2014; SAHEBKAR *et al.,* 2015).

O RSV foi isolado pela primeira vez em 1940 de raízes de *Veratrum grandiflorum* e, posteriormente, em 1963, de raízes de *Polygonum cuspidatum,* atraindo pouco interesse dos pesquisadores. Em 1992, no entanto, Siemann e Creasy reportaram que o RSV seria o responsável pelos efeitos cardioprotetores do vinho tinto. Desde então, numerosos estudos têm reportado que o RSV pode prevenir ou diminuir a progressão de hipertensão, aterosclerose, obesidade, diabetes, câncer, doença de Alzheimer, entre outras (BAUR; SINCLAIR, 2006).

Estudos *in vitro* mostraram resultados promissores no uso do RSV como antioxidante, antiagregante plaquetário e anti-inflamatório. Além disso, o RSV emergiu como importante candidato a agente antidiabético, uma vez que o mesmo é capaz de reduzir a hiperglicemia e aumentar a sensibilidade à insulina em humanos. Curiosamente, em experimentos com células isoladas, o RSV foi capaz de estimular a captação de glicose na ausência de insulina (HAUSENBLAS *et al.,* 2015; WICIŃSKI *et al.,* 2018*).*

Os efeitos benéficos da atividade do RSV possivelmente resultam de sua capacidade de neutralizar EROS (MAHAL; MUKHERJEE, 2006), de inibir a COX (BAUR, SINCLAIR; 2006) e de ativar vias anti-inflamatórias (FRÉMONT, 2000). O RSV induz aumento no nível da sirtuína-1, interrompendo a cascata TLR4/NF-κB/STAT e reduzindo a produção de citocinas na micróglia ativada (CAPIRALLA et al., 2012). Alarcon e Villega (2005) demonstraram que o RSV foi capaz de atenuar fatores pró-inflamatórios derivados de macrófagos/mastócitos, como o TNF-α, a histamina e o fator ativador de plaquetas (PAF).

Shi *et al.* (2018), ao avaliarem camundongos que receberam três doses da combinação de docetaxel, adriamicina e ciclofosfamida uma vez por semana, durante três semanas, e tratados com RSV na dose de 100 mg/Kg, diariamente por via oral durante o período, observaram que os animais que receberam RSV apresentaram melhora no comprometimento cognitivo induzido pela quimioterapia. Descreveram também que os animais tratados com RSV apresentaram níveis significativamente menores das citocinas pró-inflamatórias TNF-α e lL-6 e maiores das citocinas anti-inflamatórias IL-4 e IL-10. Outra característica relevante para possíveis efeitos sobre o SNC é a de que o RSV atravessa a BHE (BAUR *et al.,* 2006).

Os estudos em animais oferecem a possibilidade de avaliar o comprometimento cognitivo induzido pela quimioterapia, evitando fatores potencialmente passíveis de causar erro e que tanto podem comprometer a pesquisa em humanos (FARDELL *et al.,* 2011).

Como mencionado, atualmente não há qualquer tratamento reconhecido para os efeitos colaterais neurotóxicos de alguns agentes quimioterápicos (JANELSINS *et al.,* 2012; STONE; DeANGELIS, 2016).

O objetivo do presente estudo foi o de investigar os efeitos morfológicos e comportamentais da utilização da CUR e do RSV em associação com o quimioterápico DOX em ratos Wistar adultos, a fim de verificar se tais substâncias podem aliviar os prejuízos cognitivos e o comportamento reacional glial já descritos para a droga.

2 ARTIGO

Artigo submetido ao periódico *Research in Veterinary Science* (Qualis A1)

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Behavioral and morphological effects of resveratrol and curcumin in rats submitted to doxorubicin-induced cognitive impairment

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Highlights

Doxorubicin administration impaired short-term and long-term memory in rats, but not locomotor activity.

Expression of astrocyte GFAP and microglial Iba1 was increased by doxorubicin in the frontal cortex, hypothalamus and hipoccampus.

Resveratrol and curcumin reverted doxorubicin-induced astrogliosis and microgliosis.
Curcumin reverted short- and long-term memory deficits caused by doxorubicin.
Resveratrol reverted only the long-term memory impairments induced by doxorubicin.

Abstract

Doxorubicin (DOX) is known to cause cognitive impairments in patients submitted to long-term chemotherapy (deficits also known as chemobrain). Therefore, there is an urgent need for therapeutic strategies capable of returning cancer survivors back to their previous quality of life. The present study investigated whether resveratrol (RSV) or curcumin (CUR) administration could affect mnemonic function and brain morphological changes following DOX administration in rats. Male Wistar rats were divided into 4 groups: DOX group (2.5 mg/kg/week for 4 weeks, i.p., plus distilled water for 28 days, oral gavage - OG), DOX+RSV group (DOX, 2.5 mg/kg/week for 4 weeks, i.p., plus RSV, 10 mg/kg/day for 28 days, OG), DOX+CUR group (DOX, 2.5 mg/kg/week for 4 weeks, i.p., plus CUR, 100 mg/kg/day for 28 days, OG) and control (CTR) group (0.9% saline solution/week for 4 weeks, i.p., plus distilled water for 28 days, OG). Behavioral analyses (open field - OF - and the novel object recognition test - NORT) were performed. Brains were collected and analyzed by hematoxylin-eosin and luxol fast blue staining techniques and by immunohistochemistry for GFAP (glial fibrillary acidic protein) expression in astrocytes and Iba1 (ionized calcium-binding adaptor molecule 1) expression in microglia. DOX-injected rats presented short-term and long-term memory impairments as seen in the NORT at 3 and 24 hours after habituation and increased GFAP and Iba1 expression, respectively, in astrocytes and microglia of the frontal cortex, hypothalamus and hippocampus. Such cognitive deficits were reverted by CUR at both periods and by RSV at 24 hours. DOX-induced astrogliosis and microgliosis were reverted by RSV and CUR. No signs of demyelination or neuronal loss were found in any group. Thus, CUR and RSV reverted memory loss, astrogliosis and microgliosis induced by DOX monotherapy.

Keywords: astrocytes; chemotherapy; glial fibrillary acidic protein; memory impairment; microglia.

1. Introduction

Chemotherapy-induced cognitive impairment, also known as chemobrain or chemofog, is widely recognized as a frequent adverse side effect following the administration of chemotherapeutic drugs (Asher and Myers, 2015; Taillibert et al., 2016). Memory impairments have been extensively reported after chemotherapeutic protocols in murine models (Konat et al., 2008; Kitamura et al., 2015; Ramalingayya et al., 2016; Barry et al., 2018; Cardoso et al., 2020) and cognitive studies in humans (Falleti et al., 2005, Ahles and Saykin, 2007; Vardy and Tannock, 2007; Asher and Myers, 2015) and have been atributted mainly to oxidative damage (Aluise et al., 2010; Gaman et al., 2016; Keeney et al., 2018; Liao et al., 2018; Ren et al., 2018) and/or neuroinflammation (Cheung et al., 2013; Tien et al., 2016; Wang et al., 2015, 2016; Orchard et al., 2018; Shi et al., 2018).

Doxorubicin (DOX), a leading member of the anthracycline family, acts as a topoisomerase II interactive agent that is commonly employed for treating several types of solid and hematological tumors in human and veterinary oncology (Sinha and Mason, 2015).

Emerging pharmacotherapeutic approaches to the treatment of cognitive dysfunction experienced by cancer survivors and recognized in murine models include modafinil, methylphenidate, lithium, donezepil, memantine, resveratrol (RSV), curcumin (CUR), peroxisome proliferator-activated receptors (PPARs) agonists, angiotensin receptor blockers (ARBs) and nonsteroidal anti-inflammatory drugs (NSAIDs), among many others (Davis et al., 2013).

CUR [diferuloylmethane or 1,7-bis-(4-hydroxy-3-methoxyphenol)-1,6-heptadiene-3,5-dione] is the major yellow-orange pigment of turmeric, a common spice and coloring agent derived from the rhizome of the East Indian plant *Curcuma longa*, which exhibits antioxidant, anti-inflammatory, anti-cancer, neuroprotective and anti-fibrotic effects (Hsu and Cheng, 2007; Aggarwall and Harikumar, 2009; Srivastava et al., 2011; Metzler et al., 2013; Bondan et al., 2017; Liu et al., 2018).

Antioxidant, anti-inflamatory, neuroprotective, anti-carcinogenic, as well as cardioprotective actions, were also described for RSV (trans-3,4`,5-trihydroxystilbene), a naturally occurring phenolic compound found in the skin of red grapes, red wine, peanuts and cranberries (Baur and Sinclair, 2006; Saiko et al., 2008; Gupta et al., 2012; Orsu et al., 2013; Zao et al., 2013; ElBatsh and Shehata, 2015).

Prominent astrogliosis (Babets et al., 2016; Cardoso et al., 2020) and microgliosis (Allen et al., 2019) were described in the brain with DOX monotherapy. As DOX presents a poor penetration into the brain, it was hypothetized that its administration could induce a systemic inflammatory response with the increase of proinflammatory cytokine crossing through the blood-brain barrier (BBB). Into the brain, these cytokines could be involved in the elevation of oxidative molecules and proinflammatory cytokines that, in conjunct, induced glial activation (Allen et al., 2019; Cardoso et al., 2020).

Astrocytes are the most numerous glial cells in the central nervous system (CNS) performing many critical functions in health and disease conditions. In pathological conditions they quickly react to changes in the neural microenvironment by developing morphological and functional adjustements that affect neuronal activity (Sofroniew and Vinters, 2010). In response to CNS insults, astrocytes develop a hypertrophic or reactive status called astrogliosis (Sofroniew, 2009, 2015; Sofroniew and Vinters, 2010), in which increased expression of specific structural proteins occurs, such as vimentin and glial fibrillary acidic protein (GFAP) (Middeldorp and Hol, 2011; Brenner, 2014; Yang and Wang, 2016).

As the immune-competent cells of the CNS, microglia play an important role in defense and repair. In situations of injury or disease, ramified microglia rapidly transform themselves from a resting state to an active phenotype, showing rapid proliferation and migration, releasing a wide range of cytokines and becoming phagocytic (Wolf et al., 2017; Prinz et al., 2019). Ionized calcium binding adaptor molecule (Iba1) is a microglia/macrophage specific calcium-binding protein, which presents actin-bunding activity and participates in membrane ruffling and phagocytosis in activated microglia (Keiko et al., 2004).

In such context, the objective of the present study was to investigate if RSV or CUR could revert the behavioral and morphological changes found in rats submitted to a 4-week treatment with this chemotherapeutic agent, aiming to identify possible agents that could prevent or minimize cognitive deficits and glial changes induced by DOX.

2. Methods

2.1. Animals and groups

The animal procedures were performed in accordance with the guidelines of the Ethics Committee on Care and Use of Laboratory Animal Resources of University Paulista, which approved the study protocol (CEUA/ICS/UNIP, protocol no. 4608170719). All efforts were made to minimize the suffering of the animals.

A total of 40 male Wistar rats, 3 months of age and weighing 290 ± 20g, were housed in polypropylene cages (38 cm × 32 cm × 16 cm; 4 rats per cage) at a controlled temperature (22 °C ± 2 °C) and humidity (55–65%) with artificial lighting (12 h/12 h light/dark cycle, lights on at 7:00 AM). The animals had free access to Nuvilab rodent chow (Nuvital, São Paulo, SP, Brazil) and filtered water. Sterilized and residue-free wood shavings were used for animal bedding. All experiments, including treatments and behavioral observations, were performed between 9:00 AM and 12:00 AM to minimize the effects of circadian rhythms. Rats were randomly divided into 4 groups – (1) DOX group (n=10) consisting of rats receiving doxorubicin (Fauldoxo® 10 mg, 2 mg/mL, Libbs, Embu, São Paulo, Brazil) by intraperitoneal (i.p.) route with a weekly dose of 2.5 mg/kg for 4 weeks, plus distilled water for 28 days by oral gavage - OG; (2) DOX+RSV group (n=10) of rats injected with DOX and treated with resveratrol for 28 days (10 mg/kg/day, OG; 2 mg/mL in Tween 80 9%, Sigma- Aldrich, R5010, St. Louis, MO, USA); (3) DOX+CUR group (n=10) of rats injected with DOX and treated with curcumin for 28 days (100 mg/kg/day, OG; 40 mg/mL in 9% ethanol solution, Sigma-Aldrich, C1386, St. Louis, MO, USA); and (4) control (CTR) group (n=10) of rats weekly injected with the same volume of 0.9% sterile saline solution i.p., plus distilled water by OG for the same period. Experimental design diagram is shown in Figure 1.

2.2. Behavioral tests

The open field (OF) test was performed on the 6th day of the last administration of DOX or saline solution (i.e., on day 27). The novel object recognition task (NORT) was performed on day 27 for short-term memory assessment and on day 28 for long-term memory assessment (Mathiesen and DiCamillo, 2010; Vogel-Ciernia and Wood, 2014).

2.2.1. Open field (OF) test

The OF test is used to evaluate motor/exploratory behaviors and was performed as previously described (Prut and Belzung, 2003). The following parameters were recorded over 5 min: total locomotion (one unit was defined as the animal entering one square of the floor with all four paws), and rearing frequency (one unit was defined as the animal standing upright on its hindlimbs).

2.2.2. Novel object recognition test (NORT)

Rats were placed for 5 minutes in an arena in order to perform the recognition task of new objects with the same color, size, shape, and relatively heavy to prevent animals from moving them around. Interaction with such objects in this first test was employed to ensure that there would be no intrinsic preferences or aversions and that each object would be explored equally, for similar durations (habituation phase). For the assessment of short-term and long-term memory (Vogel-Ciernia and Wood, 2014), the animals were placed again in the OF arena, respectively, at 3 and 24 hours from habituation, in the presence of three objects, one of them being new to the animal (with different color, shape and size from the other two objects). It was considered as exploration the act of directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose or mouth (Mathiesen and DiCamillo, 2010; Sik et al., 2014). Each session was videotaped for 5 minutes and then analyzed. The evaluated parameters were interaction time with the new object (in seconds) and interaction time with the old object (in seconds) and the discrimination index, calculated as the difference in the exploration time for the new object compared to the old one as a proportion of the total time spent exploring the two objects (Okuda et al., 2004).

2.3. Sample collection and processing procedures

Imediately after performing the NORT, 10 rats from each group were anesthetized with thiopental (50 mg/kg, i.p.) and then submitted to intracardiac perfusion with buffered 10% formaldehyde solution. The brains were removed and fixed in 10% buffered formalin for 72h for morphological studies. Samples of the lungs, heart, liver, kidneys, spleen, stomach, gut and adrenals were also collected as parameters of general physiological conditions upon DOX treatment. Coronal sections of each brain were made to reach the frontal cortex, hippocampus and hypothalamus. The tissue was embedded in paraffin for processing for conventional histological procedures and the hematoxylin-eosin staining technique was performed. Luxol fast blue staining technique was used for myelin assessment in those different investigated brain areas.

2.3.1. GFAP and Iba1 immunohistochemistry and morphometry

Immunohistochemistry was performed using the chain polymer-conjugated staining method (DAKO EnVision System). Polyclonal rabbit anti-GFAP immunoglobulin (1:50; Z033401, Dako, Glostrup, Denmark) and polyclonal goat anti-Iba1 immunoglobulin (1:100; ab5076, abcam, Cambridge, MA, USA) were used as the primary antibodies followed by the EnVision+ Kit for detection (HRP. Rabbit. DAB+, K4011, Dako, Glostrup, Denmark). Ten sections (5 µm thick) per rat were made from each chosen area (frontal cortex, hippocampus and hypothalamus), and, from each individual section, ten photomicrographs were taken (40x objective, Nikon E200 microscope, equipped with a Nikon Coolpix digital camera linked to a liquid crystal display monitor, Kanagawa, Japan). Morphometric analysis was performed using the Image Pro-Plus 6 software (Media Cybernetics, Rockville, MD, CA, USA), calibrated with digital color filters regulating red, green and blue bits, in such a way that only positive cells were included and the background staining was excluded from the measurement. We used the astrocyte/microglia index per area (zero, as the complete absence of staining; and 1, as the total staining of the area) to represent the GFAP or Iba1 staining extent in comparison to the total area of the image.

2.6. Statistical analysis

Homoscedasticity was verified using an F-test or Bartlett´s test. Normality was assessed using the Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) followed by Tukey´s test was used to analyze OF results and morphometric data. Two-way ANOVA followed by Bonferroni´s test was used to analyze interaction time and Mann-Whitney U-test for the discrimination index in NORT. Results were expressed as means ± standard errors of the means (SEM) and, for the discrimination index, as percentage. In all cases, results were considered significant at $p < 0.05$. Statistical analyses were performed with Prism 6 software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Behavioral tests

No differences between groups were noted in relation to OF parameters (locomotion and rearing frequencies) at the end of the 4th week (Fig. 2). After the habituation period of 3 hours, NORT results clearly showed that DOX-treated rats interacted less with the new object compared to the CTR group ($p < 0.0001$; Fig. 3). The same pattern was observed with NORT performed 24 hours after habituation ($p < 0.0001$; Fig. 3). Interestingly, NORT data also showed that DOX-treated rats did not alter their interaction time with a new or previously explored object in the OF, in contrast with CTR group results after 3 and 24 hours from habituation ($p < 0.0001$, for both periods). The discrimination index clearly showed these differences between DOX and CTR groups at 3 and 24 hours (respectively, $p < 0.0001$ and $p < 0.01$; Fig. 4).

Additionally, rats from the DOX+RSV group did not interact more with the new object after 3 hours, unlike the CTR group, although they spent more time interacting with the new object at 24 hours ($p < 0.0001$; Fig. 3).

As for the DOX+CUR group, interaction time with the new object was longer at 3 hours ($p < 0.01$) and 24 hours ($p < 0.001$; Fig. 3).

Fig. 4 also shows that, at 3 hours after habituation, discrimination indexes were different between DOX+RSV and DOX+CUR groups ($p < 0.0001$), as well as between DOX and DOX+CUR groups ($p < 0.0001$). At 24 hours, differences

were found comparing DOX group vs. DOX+RSV group ($p < 0.01$) and DOX group vs. DOX+CUR group ($p < 0.05$).

3.2. Morphological observations

At light microscopy, no signs of neuronal degeneration or myelin loss were found. Although no mortality was observed in DOX groups or in the CTR group, DOX-treated rats presented at necropsy a slight enlargement of the liver. DOX treatment caused hydropic degeneration in hepatocytes in a diffuse pattern, mostly in the centrilobular zone, associated with rarefied areas of the liver parenchyma. Glomerular atrophy and diffuse tubular vacuolization in the kidney, cellular rarefaction in the white and red pulp compartments of the spleen and multifocal vacuolization in zona fasciculata of the adrenal cortex were also observed in the DOX-treated rats. No histopathological changes were seen in the heart, lungs, stomach and gut.

All investigated brain areas in the DOX-treated group (frontal cortex, hippocampus and hypothalamus) presented increased expressions of GFAP ($p < 0.0001$; Fig. 5) and Iba1 ($p < 0.0001$; Fig. 7) compared to the other groups. Photomicrographs of the GFAP immunohistochemical staining of astrocytes and of Iba1 microglial expression in the distinct groups, the groups treated with RSV and CUR reversed astrogliosis and microgliosis, are shown, respectively, in Fig. 6 and 8.

4. Discussion

Memory impairments have been extensively described after chemotherapeutic protocols in experimental murine models (Konat et al., 2008; Kitamura et al., 2015; Ramalingayya et al., 2016; Barry et al., 2018; Cardoso et al., 2020) and human studies (Falleti et al., 2005, Ahles and Saykin, 2007; Vardy and Tannock, 2007; Asher and Myers, 2015), being atributted to oxidative damage (Aluise et al., 2010; Gaman et al., 2016; Keeney et al., 2018; Liao et al., 2018; Ren et al., 2018) and/or neuroinflammation (Cheung et al., 2013; Tien et al., 2016; Wang et al., 2015, 2016; Orchard et al., 2018; Shi et al., 2018).

Contrarily to other studies (Azariova et al., 2014; Kitamura et al., 2015), no anxiety-like behavior or diminished exploratory response were observed in our

investigation regarding DOX administration. However, cognitive dysfunction was noted in the NORT performed after 3 and 24 hours from habituation, suggesting that both short-term and long-term discriminative memories were impaired.

Preventive effects of CUR against chemotherapy-induced myelosuppression, gastrointestinal toxicity, cardiotoxicity, hepatotoxicity, nephrotoxicity, neurotoxicity, ototoxicity and genotoxicity were already reported (Liu et al., 2018; Mohajeri and Sahebkar, 2018). In our study, CUR reverted memory deficits induced by DOX as shown in the NORT at 3 and 24 hours. These findings are in accordance with those from clinical studies in elderly people which indicate that CUR could attenuate cognitive deficits (Cox et al., 2015), as well as in cisplatin-treated mice, in which it was observed that cognitive deficits were restored, improving both neurogenesis and synaptogenesis, enhancing hippocampal autophagy and suppressing apoptosis (Yi et al., 2020).

As inflammation and autophagy are interrelated, the increased autophagic degradation of cellular constituents could eliminate dysfunctional or damaged mitochondria, thus counteracting cellular degeneration and attenuating inflammation (Yi et al., 2020).

CUR activates the AMP-activated protein kinase (AMPK) - C-Jun N-terminal kinase (JNK) signaling pathway, which mediates both Bcl-2 upregulation and mTOR inhibition and in turn suppresses apoptosis and enhances autophagy, respectively (Yi et al., 2020).

CUR was also shown to protect mitochondrial function from chemotherapy-induced oxidative stress. Ortega-Dominguez et al. (2017) showed thar CUR decreased cisplatin-associated damage to mitochondria through decreasing reactive oxygen species (ROS) production, as well as the increase of NAD^+-dependent deacetylase sirtuin-3 (SIRT3) and mitophagy associated proteins, thus regulating mitochondrial bioenergetics and redox balance.

Thus, both anti-inflammatory and antioxidant effects of CUR could explain the reversal of memory deficits seen in DOX-treated rats when CUR was concomitantly administered.

As for RSV administration in DOX-treated rats, NORT results showed that memory deficits were reverted only at 24 hours following habituation, but not at 3 hours, suggesting that the short-term memory impairment caused by the

alkylating agent remained. It is known that short-term and long-term memory circuits involve different neurotransmitter systems and distinct signal transduction cascades (Izquierdo et al., 1999; Vianna et al., 2000). Many treatments with specific molecular actions given into the hippocampus, entorhinal or parietal cortex after one-trial avoidance learning can effectively cancel short-term memory without affecting long-term memory formation. Nevertheless, other treatments influence both memory types similarly (Vianna et al., 2000).

So, it was established that short-term memory is not just a step towards long-term memory, but a separate entity. The anterolateral prefrontal cortex is crucial for working memory and long-term memory, but is not involved in short-term memory. The hippocampus, entorhinal and parietal cortex are crucial for the three types of memory, in some cases using different receptors for each. In its turn, the amygdala is not involved in working memory or short-term memory, but it plays a key role in the modulation of the early phase of long-term memory (Izquierdo et al., 1999, 2002).

Short-term memory do not include gene expression or protein synthesis; long-term memory include a double peak of cAMP-dependent protein kinase activity, accompanied by the phosphorylation of CREB, and both gene expression and protein synthesis. Possible cellular and molecular events that do not require mRNA or protein synthesis should account for short-term memory. These might include a hyperactivation of glutamate AMPA receptors, ribosome changes, or the exocytosis of glycoproteins that participate in cell addition (Izquierdo et al., 2002).

ElBatsh and Shehata (2015) described a protective effect of RSV on cognition in cisplatin-treated rats, restoring memory deficits 4 hours after habituation in the NORT, probably by increasing brain-derived neurothrophic factor (BDNF) levels in the hippocampus. RSV administration also ameliorated chemotherapy-induced cognitive impairments in mice that reveived a combination of docetaxel, adriamycin and cyclophosphamide (DAC protocol) (Shi et al., 2018). RSV caused in DAC-treated animals lower levels of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) and higher levels of the anti-inflammatory cytokines IL-4 and IL-10 in both serum and brain. In this context, RSV appeared to modulate the cytokine-

regulating pathway peroxisome proliferator activated receptor (PPAR) - γ/ nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and also protected against DAC-induced decreases in the expression of several neuroplasticity biomarkers, including BDNF (Shi et al., 2018).

Chemotherapy with methotrexate was associated with persistent tri-glial dysregulation in mice submitted to long-term administration of this drug, inducing a persistent activation of microglia and subsequently of astrocytes, which was dependent on inflammatory microglia (Gibson el al., 2019).

Geraghty et al. (2019) reported that activated microglia induced cortical neurons to reduce BDNF expression, which was restored by microglial depletion, and low BDNF levels reduced myelination by oligodendrocytes, triggering the memory losses associated to methotrexate chemotherapy.

Overexpression of Iba1 (Allen et al., 2019) and of GFAP (Cardoso et al., 2020) is described in several brain areas following DOX treatment, suggesting, respectively, the occurrence of a strong microglial and astrocyte response, due to the neuroinflammatory response induced by this agent. Increased brain levels of the pro-inflammatory cytokines TNF-α (Tangpong et al., 2006; Keeney et al., 2015), IL-6, IL-8 and CXCL1 (Cardoso et al., 2020) were found in murine models of DOX chemotherapy, along with a decrease of the anti-inflammatory IL-10.

Allen et al. (2019) proposed that attenuation of neuroinflammation and restoration of microglia normal function could ameliorate the adverse neurocognitive effects of DOX use.

As CUR and RSV present anti-inflammatory actions, the reversal of DOX-induced microgliosis and astrogliosis was expected. For instance, Cheng et al. (2015) found that increasing concentrations of RSV could decrease in a dose-dependent manner GFAP expression of hippocampal astrocytes in an Alzheimer's disease model in rats, also reducing TNF-α levels. CUR also decreased markedly the peripheral astrogliosis around the lesion site in a model of gliotoxic injury in the rat brainstem (Bondan et al., 2017). Iba1 expression was reduced by RSV (Gatson et al., 2013; Pan et al., 2016; Yang et al., 2016; Feng and Zhang, 2019; Yan et al., 2019) and CUR (Kau et al., 2015; Liu et al., 2017; Ullah et al, 2020) in different situations of glial activation and distinct models of neuroinflammation.

It has been increasingly recognized that glial dysfunction following injury can alter mechanisms of synaptic plasticity and may be related to an increased risk for persistent memory deficits (Sajja et al., 2016). Furthermore, it has been shown that, by blocking microglial activation, cognition could be improved (Bedi et al., 2013).

Current views suggest that astrocytes are critical for higher brain functions, including learning and memory, once (1) they are excitable cells that respond to neurotransmitters released at synapses; (2) they communicate with each other, releasing their own gliotransmitters, which are essential for synaptic plasticity; (3) they synchronize neuronal activity and activate or inhibit complete neuronal networks; and, finally, (4) they release lactate in an activity-dependent manner in order to supply neuronal metabolic demand (Moraga-Amaro et al., 2014). It is known, for instance, that astrocytic lactate is necessary for the formation of long-term memory (memory consolidation), but not for short-term memory (Suzuki et al., 2011).

Many preclinical and neuroimaging studies have provided a potential link between the neurotoxic effects of chemotherapy and hippocampal dysfunction with associated learning and memory deficits (Dietrich et al., 2015) as observed in DOX-treated rats from our study.

Taken together, our findings implicate hippocampal astrogliosis and microgliosis as possible causal factors in inducing chemobrain. It is recognized that the hippocampal-amygdala circuit is disrupted following chemotherapy and the strategy of re-switching the chemo-injured CNS microenvironment to a less inflammatory state could promote recovery from chemobrain (Allen et al., 2019).

Astrocytes and microglia exert a multitude of essential functions including major roles in homeostasis maintenance and innate immune response within the CNS (Kim, de Vellis, 2005; Sofroniew and Vinters 2010; Sofroniew, 2015; Wolf et al., 2017; Prinz et al., 2019). When responding to exogenous or endogenous signals, both astrocytes and microglia adopt an activated phenotype resulting in the release of pro-inflammatory mediators. This defense system, known as neuroinflammation, is essential in normal tissue repair and in defense against external pathogens invasion. However, this process, when persistent, can

become deleterious through the release of neurotoxic factors that amplify underlying disease (Tjalkens et al., 2017).

Thus, there is a general agreement that sustained neuroinflammation is responsible for maintaining long-term cognitive dysfunctions in aging and neurodegenerative diseases (Glass et al., 2010; Michaud et al., 2013).

Activated glia release a great variety of inflammatory factors including cytokines, chemokines, ROS, and nitric oxide (NO) that are toxic to neurons (Kim and de Vellis, 2005; Sofroniew, 2009; Sofroniew and Vinters, 2010). Cytokines such as TNF-α and IL-6 are upregulated very quickly in activated glial cells and can directly amplify neuroinflammation through recruitment of both innate and adaptive immune cells and additional glial hyperactivation, leading to neuronal apoptosis (Tjalkens et al., 2017).

Inflammatory activation of glial cells is regulated by several different pathways including mitogen-activated protein kinases (MAPKs), activator protein-1 (AP-1), Janus kinase (JAK) / signal transducer and activator of transcription (STAT), and interferon regulator factor families (Glass et al., 2010); nevertheless, the NF-κB route appears to be the primary pathway involved in the activation of pro-inflammatory genes (Karin, 2005; Tjalkens et al., 2017).

In such context, glial cells appear to be not only critical sensors of local CNS health and disease states, but also cellular effectors that could create a distinct environment within the brain affecting neuronal function and, thus, animal behavior (Gibson et al., 2019; Gutmann, 2019). The finding that chemotherapy results in the establishment of a distinct functional state for astrocytes and microglia reinforces the concept that glial cells are highly dynamic cell populations whose biology is mostly dictated by the local microenvironment (Gutmann, 2019) and allows the search for new therapeutic approaches for the cognitive impairments resulting from the chemotherapeutic protocols.

Conclusions

Since chemotherapy-induced cognitive impairments have been atributted to oxidative damage and/or neuroinflammation and RSV and CUR present antioxidant and anti-inflammatory actions, it was seen that both treatments were

capable of reverting glial activation (astrocytic and microglial) and memory deficits induced by DOX.

Conflict of interest

The authors have no conflict of interest to declare.

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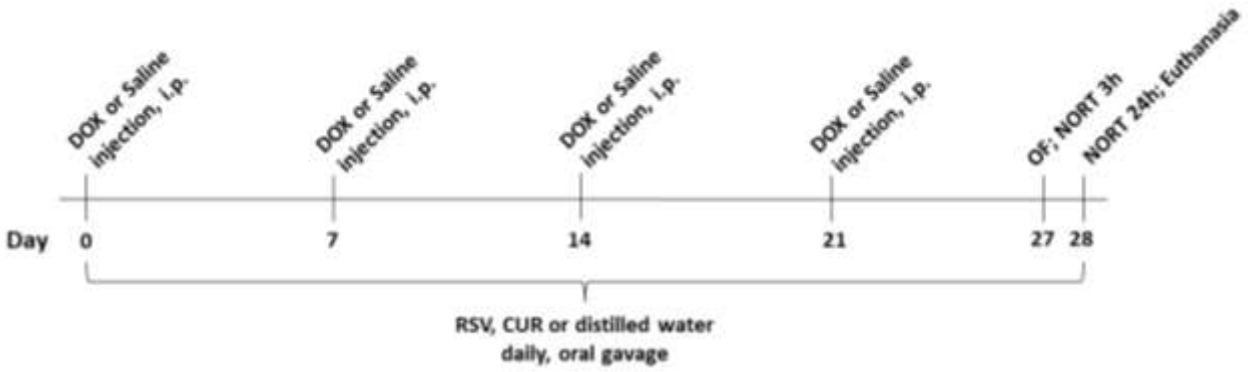

Fig. 1 - Experimental design diagram. DOX - doxorrubicin; CUR - curcumin; RSV – resveratrol; i.p. – intraperitoneal route; NORT - novel object recognition test; OF - open field test.

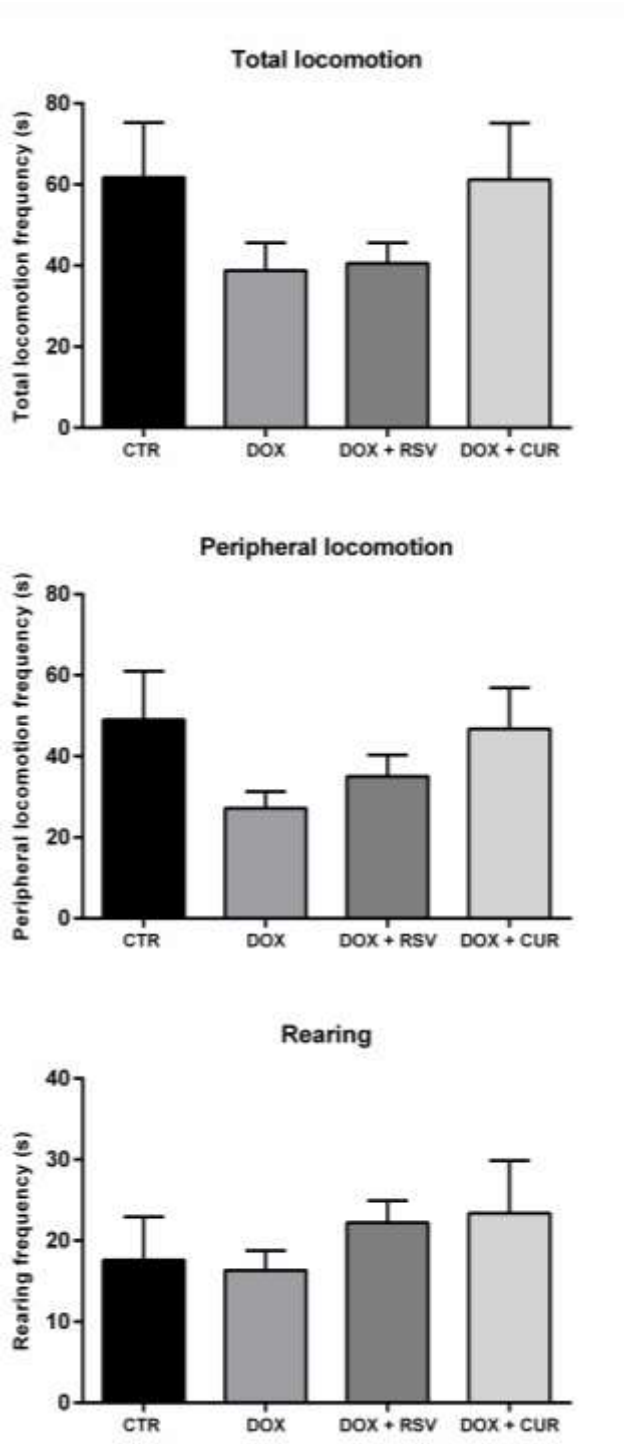

Fig. 2 - Locomotion and rearing frequencies on day 34 of the study. Control group (CTR, 0.9% saline solution once a week for 4 weeks, i.p.; distilled water for 28 days, oral gavage), doxorubicin group (DOX, 2.5 mg/kg/week, for 4 weeks, i.p.; distilled water for 28 days, oral gavage), doxorubicin plus resveratrol group (DOX+RSV, DOX, 2.5 mg/kg/week, for 4 weeks, i.p.; RSV, 100 mg/kg/day, for 28 days, oral gavage), doxorubicin plus curcumin group (DOX+CUR, DOX, 2.5 mg/kg/week, for 4 weeks, i.p., CUR, 10 mg/kg/day, for 28 days, oral gavage). Data are expressed as mean ± SEM. $p > 0.05$ (one-way ANOVA).

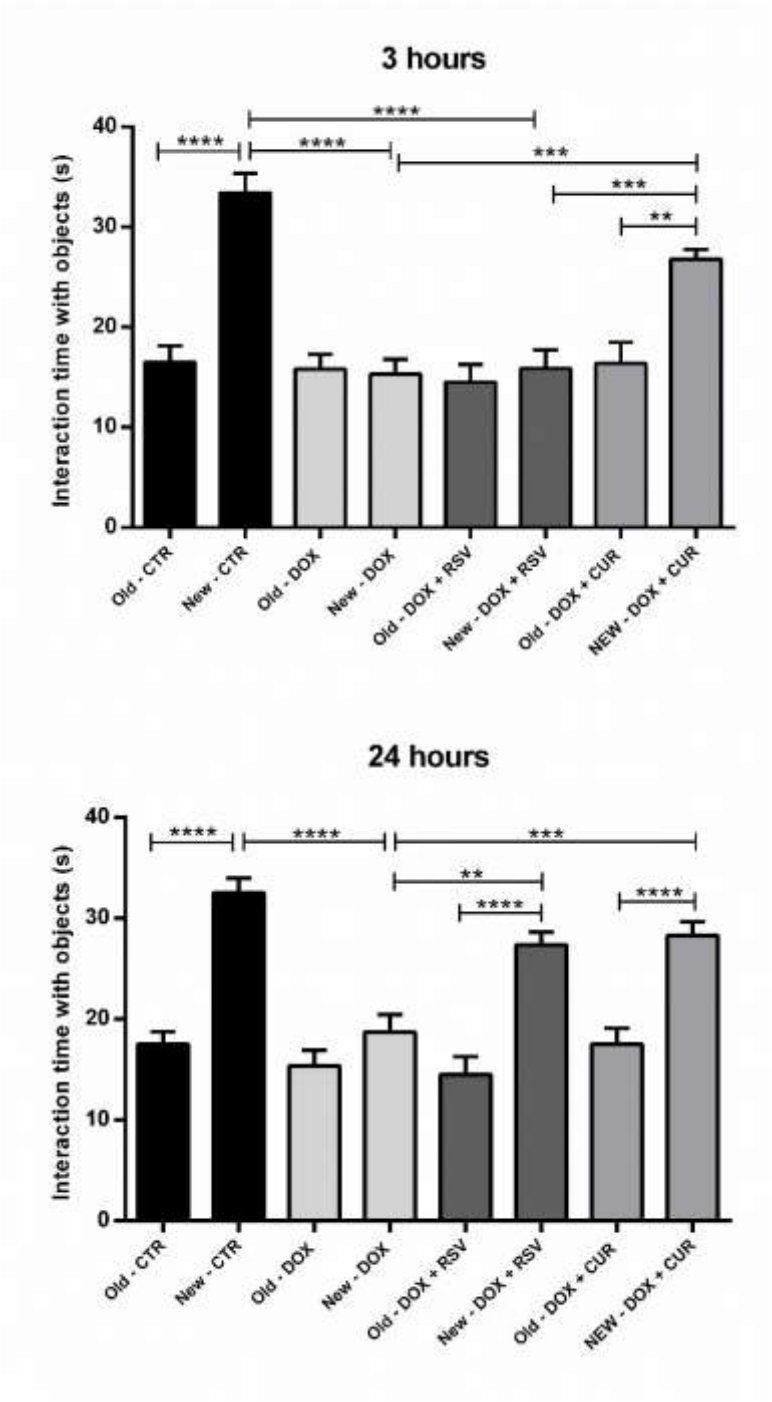

Fig. 3 - Interaction time in seconds (s) with a new or previously explored object 3h or 24h after habituation period in the novel object recognition test (NORT). Control group (CTR, 0.9% saline solution once a week for 4 weeks, i.p.; distilled water for 28 days, oral gavage), doxorubicin group (DOX, 2.5 mg/kg/week, for 4 weeks, i.p.; distilled water for 28 days, oral gavage), doxorubicin plus resveratrol group (DOX+RSV, DOX, 2.5 mg/kg/week, for 4 weeks, i.p.; RSV, 100 mg/kg/day, for 28 days, oral gavage), doxorubicin plus curcumin group (DOX+CUR, DOX, 2.5 mg/kg/week, for 4 weeks, i.p., CUR, 10 mg/kg/day, for 28 days, oral gavage). Data are expressed as mean ± SEM for interaction time. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (two-way ANOVA, followed by the Bonferroni´s test).

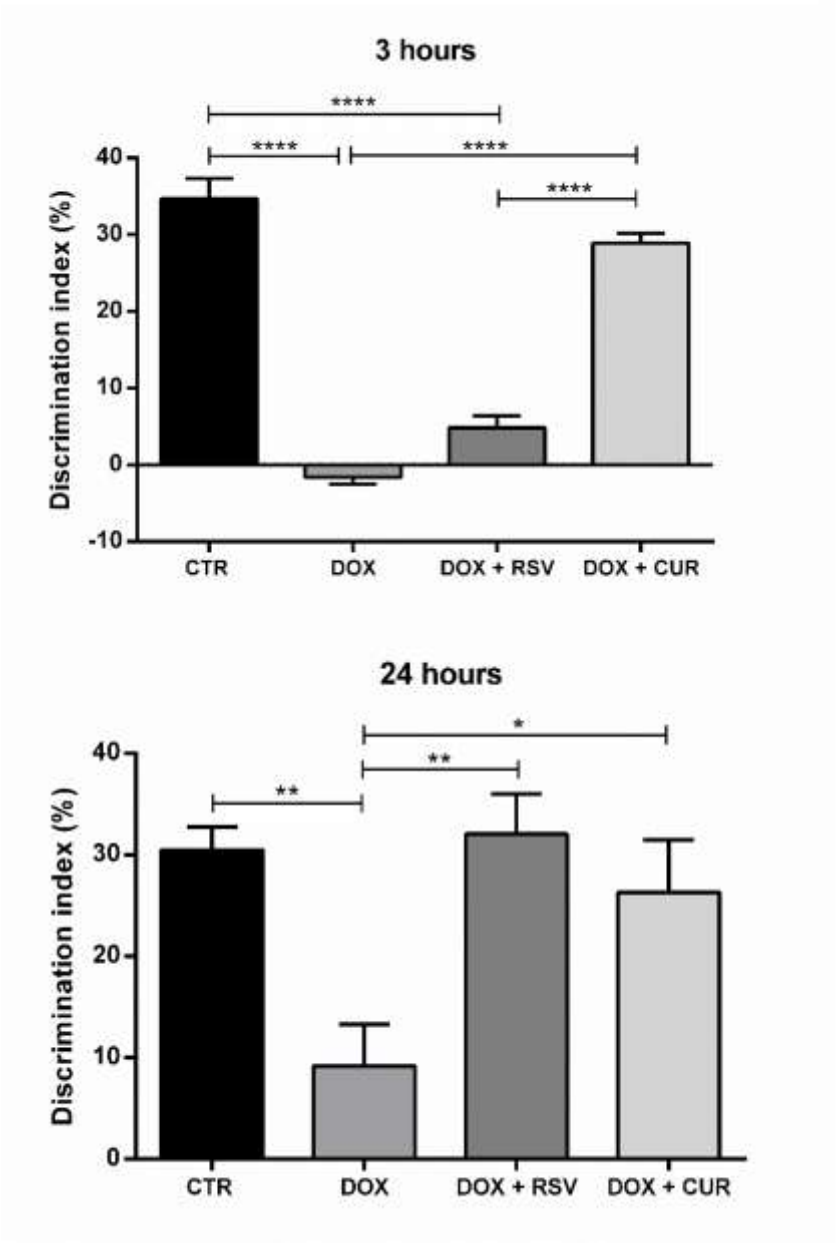

Fig. 4 - Discrimination index in the novel object recognition test (NORT). 3h or 24h after habituation period. Control group (CTR, 0.9% saline solution once a week for 4 weeks, i.p.; distilled water for 28 days, oral gavage), doxorubicin group (DOX, 2.5 mg/kg/week, for 4 weeks, i.p.; distilled water for 28 days, oral gavage), doxorubicin plus resveratrol group (DOX+RSV, DOX, 2.5 mg/kg/week, for 4 weeks, i.p.; RSV, 100 mg/kg/day, for 28 days, oral gavage), doxorubicin plus curcumin group (DOX+CUR, DOX, 2.5 mg/kg/week, for 4 weeks, i.p., CUR, 10 mg/kg/day, for 28 days, oral gavage). Data are expressed as percentage for the discrimination index. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ (Mann-Whitney U-test).

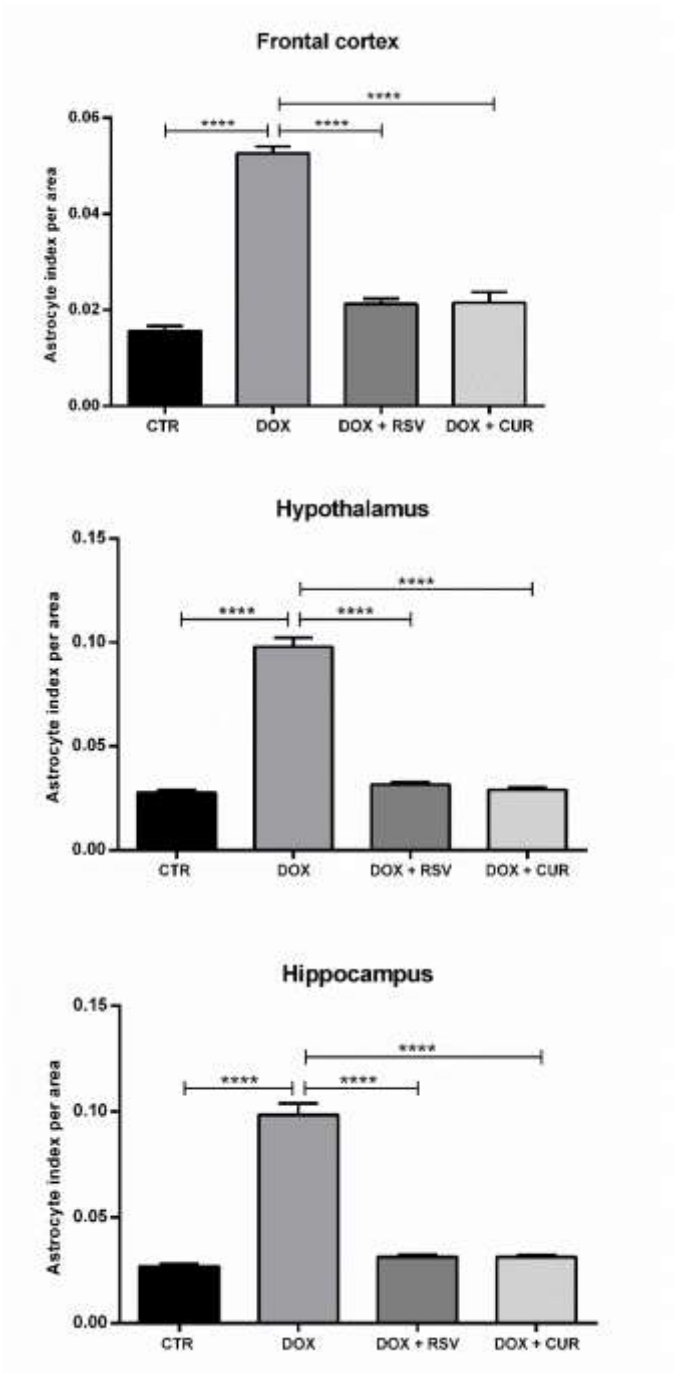

Fig. 5 - Expression of glial fibrillary acidic protein (GFAP) in the frontal cortex, hippocampus and hypothalamus of rats treated with 0.9% saline solution (once a week for 4 weeks, i.p.; distilled water for 28 days, oral gavage; control group - CTR), doxorubicin (DOX group, 2.5 mg/kg/week for 4 weeks, i.p.; distilled water for 28 days, oral gavage), doxorubicin plus resveratrol (DOX+RSV group, DOX, 2.5 mg/kg/week, for 4 weeks, i.p.; RSV, 100 mg/kg/day, for 28 days, oral gavage) or doxorubicin plus curcumin (DOX+CUR group, DOX, 2.5 mg/kg/week, for 4 weeks, i.p., CUR, 10 mg/kg/day, for 28 days, oral gavage). Data are expressed as mean ± SEM. **** $p < 0.0001$ (One-way ANOVA followed by Tukey´s test). The astrocyte index per area represents the proportion of the stained area in relation to the total area of the image, with 0 being the complete absence of staining and 1, the total staining of the area.

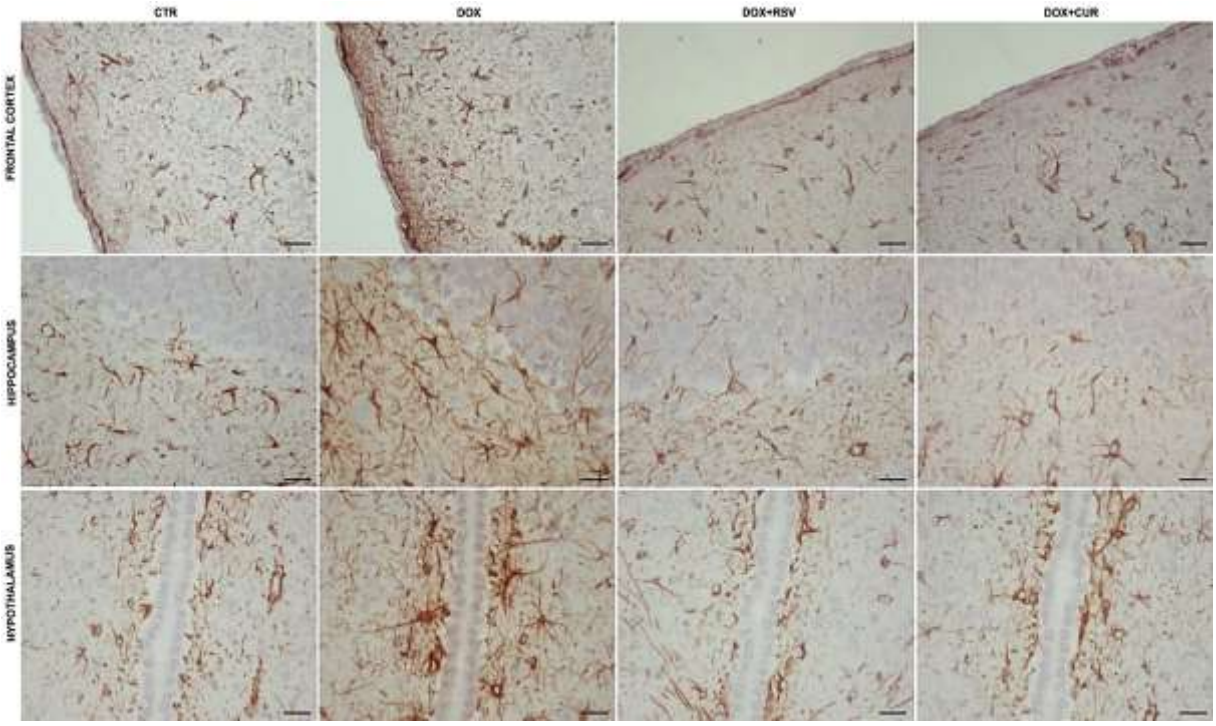

Fig. 6 - Photomicrographs of GFAP immunohistochemical staining in the frontal cortex, hippocampus and hypothalamus of rats treated treated with 0.9% saline solution (once a week for 4 weeks, i.p.; distilled water for 28 days, oral gavage; control group - CTR), doxorubicin (DOX group, 2.5 mg/kg/week for 4 weeks, i.p.; distilled water for 28 days, oral gavage), doxorubicin plus resveratrol (DOX+RSV group, DOX, 2.5 mg/kg/week, for 4 weeks, i.p.; RSV, 100 mg/kg/day, for 28 days, oral gavage) or doxorubicin plus curcumin (DOX+CUR group, DOX, 2.5 mg/kg/week, for 4 weeks, i.p., CUR, 10 mg/kg/day, for 28 days, oral gavage). Scale bar = 50 μm.

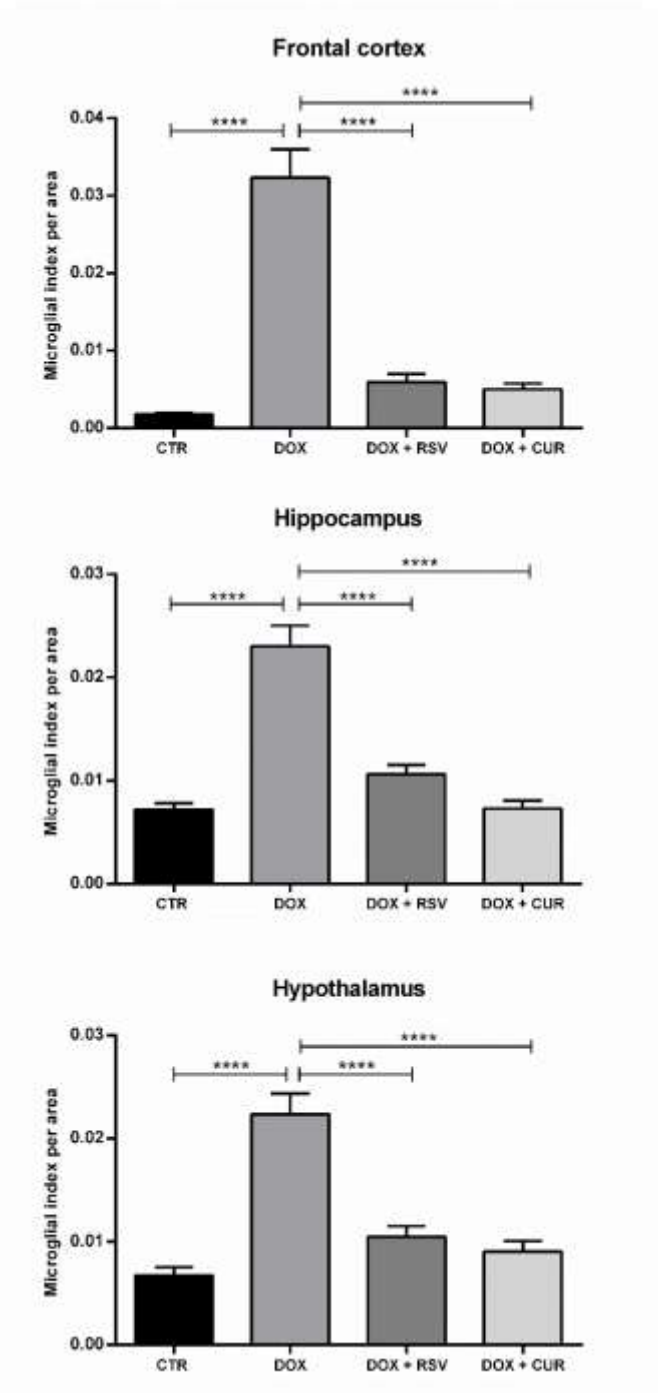

Fig. 7 - Expression of microglial Iba1 immunostaining in the frontal cortex, hippocampus and hypothalamus of rats treated with 0.9% saline solution (once a week for 4 weeks, i.p.; distilled water for 28 days, oral gavage; control group - CTR), doxorubicin (DOX group, 2.5 mg/kg/week for 4 weeks, i.p.; distilled water for 28 days, oral gavage), doxorubicin plus resveratrol (DOX+RSV group, DOX, 2.5 mg/kg/week, for 4 weeks, i.p.; RSV, 100 mg/kg/day, for 28 days, oral gavage) or doxorubicin plus curcumin (DOX+CUR group, DOX, 2.5 mg/kg/week, for 4 weeks, i.p., CUR, 10 mg/kg/day, for 28 days, oral gavage). Data are expressed as mean ± SEM. **** $p < 0.0001$ (One-way ANOVA followed by Tukey´s test). microglia index per area represents the proportion of the stained area in relation to the total area of the image, with 0 being the complete absence of staining and 1, the total staining of the area.

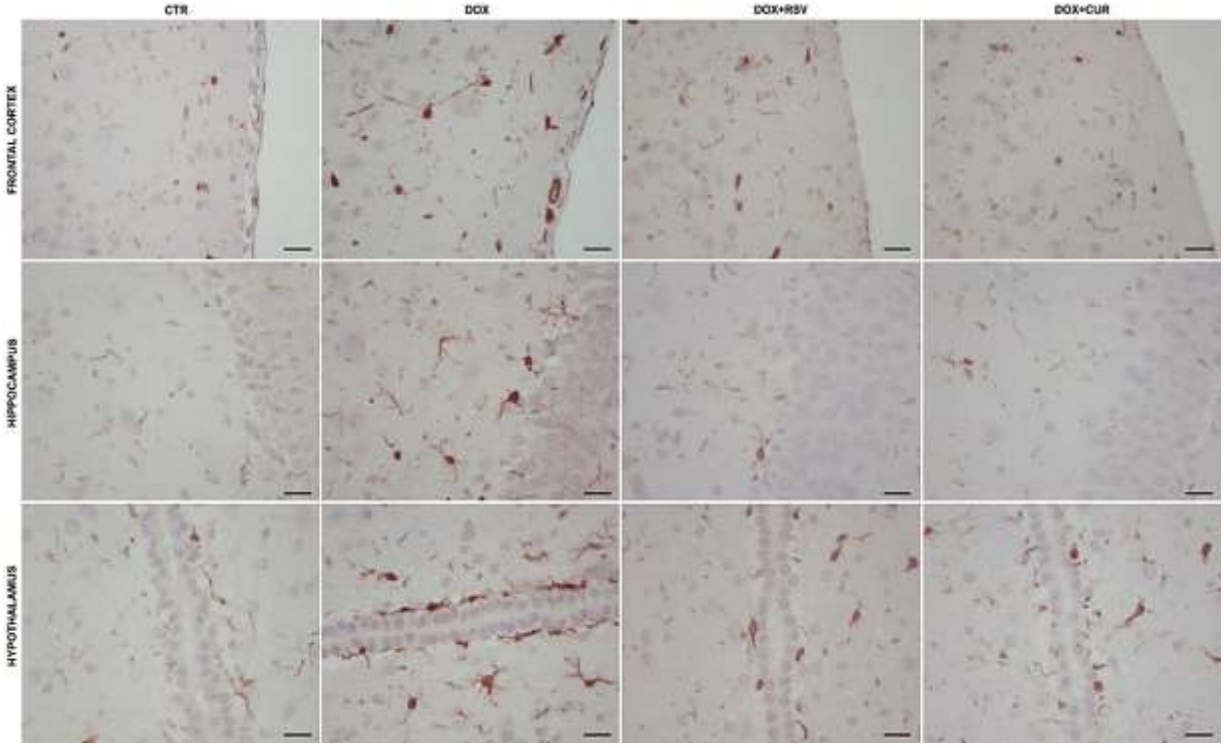

Fig. 8 - Photomicrographs of Iba1 immunohistochemical staining in the frontal cortex, hippocampus and hypothalamus of rats treated with 0.9% saline solution (once a week for 4 weeks, i.p.; distilled water for 28 days, oral gavage; control group - CTR), doxorubicin (DOX group, 2.5 mg/kg/week for 4 weeks, i.p.; distilled water for 28 days, oral gavage), doxorubicin plus resveratrol (DOX+RSV group, DOX, 2.5 mg/kg/week, for 4 weeks, i.p.; RSV, 100 mg/kg/day, for 28 days, oral gavage) or doxorubicin plus curcumin (DOX+CUR group, DOX, 2.5 mg/kg/week, for 4 weeks, i.p., CUR, 10 mg/kg/day, for 28 days, oral gavage). Scale bar = 50 μm.

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UNIP UNIVERSIDADE PAULISTA

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Estudo dos efeitos morfológicos e comportamentais da administração de doxorrubicina associada à curcumina e ao resveratrol em ratos Wistar", protocolada sob o CEUA nº 4608170719 (ID 000287), sob a responsabilidade de **Renata Larocca Moretti** *e equipe; Eduardo Fernandes Bondan; Carolina Vieira Cardoso* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Paulista (CEUA/UNIP) na reunião de 20/09/2019.

We certify that the proposal "Study of the morphological and behavioral effects of curcumin and resveratrol administration of doxorubicin in Wistar rats", utilizing 40 Heterogenics rats (40 males), protocol number CEUA 4608170719 (ID 000287), under the responsibility of **Renata Larocca Moretti** *and team; Eduardo Fernandes Bondan; Carolina Vieira Cardoso* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Paulista University (CEUA/UNIP) in the meeting of 09/20/2019.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 09/2019 a 09/2020 Área: Enfermagem

Origem: Biotério de Experimentação

Espécie: Ratos heterogênicos sexo: Machos idade: 4 a 6 semanas N: 40

Linhagem: Ratos Wistar Peso: 200 a 350 g

Local do experimento: Os experimentos serão realizados nos laboratórios de experimentação animal da Pós-Graduação em Patologia Ambiental e Experimental da Universidade Paulista.

São Paulo, 25 de novembro de 2019

Prof. Dr. Vicente Borelli
Coordenador da Comissão de Ética no Uso de Animais
Universidade Paulista

Profa. Dra. Maria Martha Bernardi
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Universidade Paulista