UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA

Alcindo Busanello

EFEITO DO RESVERATROL NAS ALTERAÇÕES COMPORTAMENTAIS INDUZIDAS POR FLUFENAZINA EM RATOS E SUA INTERAÇÃO COM A ENZIMA MONOAMINOXIDASE *in silico* e *in vitro*

Santa Maria, RS, Brasil
2016

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Tese apresentada ao Programa de Pós-Graduação em Farmacologia, Área de Concentração em Farmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Farmacologia**

Orientadora: Profª. Drª. Roselei Fachinetto
Co-orientadora: Profª. Drª. Nilda Berenice de Vargas Barbosa

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Aprovado em 11 de agosto de 2016:

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Santa Maria, RS
2016.

DEDICATÓRIA

A minha família, minha esposa e companheira Roselei e a nosso filho João Lucas.

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Agradeço a Deus, que nas suas mais diferentes formas de manifestação, está sempre presente guiando meus passos.

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Leonardo da Vinci

RESUMO

EFEITO DO RESVERATROL NAS ALTERAÇÕES COMPORTAMENTAIS INDUZIDAS POR FLUFENAZINA EM RATOS E SUA INTERAÇÃO COM A ENZIMA MONOAMINOXIDASE *in silico* e *in vitro*

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Os antipsicóticos típicos, comumente utilizados para o tratamento da esquizofrenia, causam discinesia tardia em humanos e discinesia orofacial (DO) em roedores. Neste trabalho, investigamos o efeito do resveratrol, um polifenol com propriedades neuroprotetoras encontrado principalmente em frutas vermelhas e no vinho tinto, sobre as alterações comportamentais induzidas por tratamento agudo e crônico com flufenazina em ratos. Além disso, avaliamos o efeito do resveratrol sobre a enzima monoaminoxidase (MAO) *in vitro*, *in silico* e a participação desta na DO. Com isto, o primeiro objetivo de nosso estudo foi investigar a ação do resveratrol (utilizando uma dose baixa), num modelo agudo de movimentos de mascar no vazio (MMV) induzido pela administração de flufenazina em ratos. Neste trabalho, observamos que o resveratrol, na dose de 1 mg/kg administrado 3 vezes na semana durante 21 dias, reduziu a prevalência dos MMV, mas não a intensidade da DO, representada pelo número de MMV. O tratamento com flufenazina reduziu a atividade locomotora e exploratória em campo aberto e o co-tratamento com resveratrol protegeu parcialmente. Como alguns estudos sugerem que um dos possíveis alvos do resveratrol é a enzima MAO, o segundo objetivo foi avaliar o efeito do resveratrol sobre a atividade da MAO *in vitro* e *in silico*. O resveratrol inibiu ambas isoformas da MAO, porém com uma potência aproximadamente 28 vezes maior para a MAO-A do que para a MAO-B. Os dados da análise da cinética de inibição da MAO na presença do resveratrol demonstraram que houve alteração da V_{max} sem alteração do K_m, indicando um perfil de inibição do tipo não competitivo tanto para a MAO-A quanto para a MAO-B. Além disso, um perfil parcialmente reversível para MAO-A e completamente reversível para MAO-B foi obtido. No estudo *in silico* usando a enzima humana, ambas isoformas do resveratrol interagiram com o sítio ativo da enzima evitando a entrada do substrato, o *cis*-resveratrol para a MAO-A, e o *trans*-resveratrol para a MAO-B. Os grupos hidroxila do resveratrol formando ligações de hidrogênio com a enzima podem ser os responsáveis pela afinidade entre eles. O terceiro objetivo deste trabalho foi investigar o efeito do resveratrol, administrado na água de beber, na dose de 20 mg/kg, em um modelo crônico de DO (126 dias) induzida por flufenazina em ratos, bem como avaliar se a alteração na atividade da MAO poderia estar envolvida no possível efeito protetor do resveratrol. A flufenazina reduziu o ganho de peso e a atividade locomotora e exploratória dos animais e o co-tratamento com resveratrol não alterou estes parâmetros. O tratamento crônico com flufenazina aumentou o número de MMV e o co-tratamento com resveratrol reduziu a intensidade dos MMV. No que se refere à atividade da MAO, apenas a atividade da MAO-B no estriado do grupo tratado com flufenazina foi menor em relação ao grupo resveratrol. No geral, os resultados sugerem que as doses de resveratrol testadas foram eficazes em reduzir a DO em ratos. Embora o resveratrol tenha inibido a atividade da MAO *in vitro*, é provável que seus efeitos na DO não sejam dependentes de uma ação direta sobre a atividade da enzima.

Palavras chave: Discinesia tardia. Sistema dopaminérgico. MMV. Antipsicóticos. MAO.

ABSTRACT

EFFECT OF RESVERATROL IN FLUPHENAZINE-INDUCED CHANGES IN RATS AND ITS INTERACTION WITH THE ENZYME MONOAMINOXIDASE *in silico* AND *in vitro*

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ADVISOR: Roselei Fachinetto
CO-ADVISOR: Nilda Berenice de Vargas Barbosa

Typical antipsychotics, commonly used to schizophrenia treatment, cause tardive dyskinesia in humans and orofacial dyskinesia (OD) in rodents. In the present work, we investigated the effects of resveratrol, a polyphenol with neuroprotective properties mainly found in red fruits and wine, on the behavioral alterations induced by acute and chronic treatment with fluphenazine in rats. Furthermore, we evaluated the effects of resveratrol on the enzyme monoaminoxidade (MAO) *in vitro*, *in silico* and the participation of MAO in OD. Thus, the first aim of the present study was to investigate the action of resveratrol (using a low dose), in an acute model of vacuous chewing movements (VCMs) induced by the administration of fluphenazine in rats. In this study, we observed that resveratrol, at a dose of 1 mg/kg administered 3 times a week during 21 days, reduced the prevalence of VCMs, but not the intensity of OD, represented by number of VCMs. The treatment with fluphenazine reduced the locomotor and exploratory activity in open field and the co-treatment with resveratrol protected partially. As some studies suggest that MAO enzyme is a possible target of resveratrol, the second aim was to evaluate the effects of resveratrol on MAO activity *in vitro* and *in silico*. Resveratrol inhibits both isoforms of MAO, however with a potency approximately of 28 times higher to MAO-A than MAO-B. The data from analyse of kinetic of inhibition of MAO in the presence of resveratrol showed an alteration in V_{max} without alter the K_m, indicating a non competitive profile of inhibition to MAO-A as well as MAO-B. Furthermore, a profile partially reversible to MAO-A and completely reversible to MAO-B was obtained. In *in silico* study using the human enzyme, both isoforms of resveratrol interacted with the active site of enzyme avoiding the entry of substrate, the *cis*-resveratrol to MAO-A, and the *trans*-resveratrol to MAO-B. Hydroxyl groups from resveratrol in the H-bonds of the enzymes can be responsible for the affinity with them. The third aim of this work was to investigate the effects of resveratrol, administered in drinking water at a dose of 20 mg/kg, in a chronic model of OD (126 days) induced by fluphenazine in rats, as well as evaluate if alterations on MAO activity could be involved in the possible protective effect of resveratrol. Fluphenazine reduced the body weight gain and the locomotor and exploratory activity of the animals and the co-treatment with resveratrol did not alter these parameters. The chronic treatment with fluphenazine increased the number of VCMs and the co-treatment with resveratrol reduced the intensity of VCMs. With regard to MAO, only the striatal activity of MAO-B in the group treated with fluphenazine decreased in relation to resveratrol group. In general, the results suggest that the tested doses of resveratrol were efficacious in reduce OD in rats. Besides resveratrol had inhibited the activity os MAO *in vitro*, it is probable that its effects were not dependents of a direct action of resveratrol on the activity of this enzyme.

Key words: Tardive dyskinesia. Dopaminergic system. VCM. Antipsychotics, MAO.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

5-HIAA – ácido 5-hidroxiindol acético

5-HT – 5-hidroxitriptamina

COMT – catecol-O-metiltransferase

DA – dopamina

DO – discinesia orofacial

DOPAC – ácido 3,4-diidroxifenilacético

DT – discinesia tardia

EROS – espécies reativas de oxigênio

FAD – flavina adenina dinucleotideo

GST – glutationa-S-transferase

H_2O_2 – peróxido de hidrogênio

HVA – ácido homovanílico

Hz – Hertz

K_m – Constante de Michaelis-Menten

MAO - monoaminoxidase

MMV – movimentos de mascar no vazio

MPP^+ – 1-metil-4-fenil-piridínio

$OH^{\cdot}$ – radicais hidroxila

SEP – síndrome extrapiramidal

SNC – sistema nervoso central

TDA – transportador de dopamina

TH – tirosina hidroxilase

TVMA-2 – transportador vesicular de monoaminas 2

V_{max} – velocidade máxima

SUMÁRIO

APRESENTAÇÃO

No item **INTRODUÇÃO**, está descrita uma revisão sucinta sobre os temas trabalhados nesta tese.

Os resultados que fazem parte desta tese estão apresentados sob a forma de um artigo publicado e dois manuscritos, os quais se encontram no item **RESULTADOS**. As seções Materiais e Métodos, Resultados, Referências Bibliográficas, encontram-se nos próprios artigo e manuscritos e representam a íntegra deste estudo.

O item **DISCUSSÃO** apresenta uma discussão sucinta de todos os dados encontrados.

O item **CONCLUSÕES** encontra-se no final dessa tese, e apresenta as conclusões sobre o artigo publicado e os manuscritos contidos nesse trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se às citações que aparecem nos itens **INTRODUÇÃO** e **DISCUSSÃO** dessa tese.

1. INTRODUÇÃO

1.1 ESQUIZOFRENIA

A gênese da maioria dos transtornos psiquiátricos é classificada como complexa, uma vez que os mesmos não são facilmente explicados por um único componente genético ou ambiental. Um dos mais debilitantes transtornos psiquiátricos é a esquizofrenia, a qual afeta cerca de 1% da população. Uma vez que os sintomas da esquizofrenia ocorrem (geralmente na idade adulta jovem), eles persistem por toda a vida do paciente e são para a maioria destes incapacitantes (Sawa & Snyder, 2002).

Na ausência de uma anormalidade molecular conhecida, o diagnóstico é baseado na apresentação simultânea de dois tipos de sintomas que refletem uma perturbação psicótica: sintomas "positivos" que incluem delírios, alucinações e pensamentos bizarros e, sintomas "negativos" que incluem isolamento social e afetivo, falta de motivação e apatia (Stahl 2014). O diagnóstico desta patologia é particularmente complicado, uma vez que, pacientes com distúrbios afetivos, tais como distúrbio bipolar, podem apresentar um subconjunto dos sintomas psicóticos associados com a esquizofrenia, tais como alucinações.

A complexidade da origem da esquizofrenia dificulta bastante o tratamento. No entanto, os fármacos utilizados com maior eficácia são os antipsicóticos.

1.2. NEUROLÉPTICOS OU ANTIPSICÓTICOS

Os antipsicóticos foram primeiramente utilizados na década de 50 tendo sido descobertos ao acaso quando se observou que um fármaco com atividade anti-histamínica (clorpromazina) tinha efeitos antipsicóticos quando testada em pacientes esquizofrênicos. Os antipsicóticos foram designados "neurolépticos", a partir do termo grego que significa "parar o neurônio" e por causarem "neurolepsia", uma forma extrema de lentificação ou ausência de movimentos motores, assim como indiferença comportamental, em animais experimentais (Sawa & Snyder, 2002). Esta designação foi baseada no trabalho pioneiro de Jean Delay e Pierre Deniker, onde foi observado que a dose eficaz de clorpromazina (o primeiro neuroléptico utilizado em pacientes com esquizofrenia) variou muito entre os pacientes. Além disso, eles observaram que as respostas benéficas geralmente ocorreram com doses que causaram efeitos colaterais neurológicos que se assemelham a doença de Parkinson. Devido a doença de Parkinson estar associada à degeneração dos neurônios dopaminérgicos que se projetam para os núcleos *caudado* e *putamen* do cérebro, pensou-se que os antipsicóticos "parariam" os neurônios de maneira similar. Então, através de estudos de *tournouver* da dopamina e medidas diretas de receptores de dopamina, constatou-se que os antipsicóticos bloqueiam o subtipo D_2

de receptor de dopamina (Creese et al., 1976; Seeman et al., 1976). O bloqueio dos receptores nos núcleos *caudado* e *putamen* foi associado aos efeitos colaterais dos antipsicóticos, e o bloqueio dos receptores em áreas límbicas tais como o *nucleus accumbens* e córtex pré-frontal cerebral, que regulam o comportamento emocional, com os efeitos antipsicóticos dos fármacos. Além disso, também foi demonstrado que a administração de anfetaminas, que agem através da liberação de dopamina, é um modelo para exacerbar os sintomas da esquizofrenia. Estes efeitos das anfetaminas levaram a "hipótese da dopamina" para a modulação dos sintomas da esquizofrenia, onde o excesso de dopamina culmina com a piora enquanto que uma diminuição leva ao alívio dos sintomas desta patologia (Creese et al., 1976; Seeman et al., 1976; Carlson, 1988). A partir de então, a atividade antipsicótica de outros fármacos foi descoberta, dente eles a flufenazina, os quais foram denominados de antipsicóticos clássicos ou típicos. Estes possuem como mecanismo de ação o bloqueio de receptores principalmente da classe D_2.

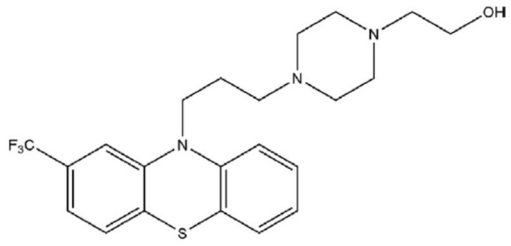
Os antipsicóticos atípicos, como a risperidona e a olanzapina por exemplo, começaram a ser sintetizados visando minimizar os efeitos colaterais extrapiramidais sem, contudo, diminuir a eficácia terapêutica. Denominam-se atípicos pelo seu menor perfil de causar síndrome extrapiramidal em relação aos típicos bem como por bloquearem além dos receptores dopaminérgicos também os receptores de serotonina. Entretanto, os antipsicóticos atípicos, além de não possuírem eficácia satisfatória nos sintomas positivos da esquizofrenia, causavam uma série de efeitos colaterais, entre eles diabetes mellitus tipo 2, agranulocitose e, em alguns casos, a própria discinesia tardia (DT) (Henderson, 2002; Kim et al., 2014). Além disso, os antipsicóticos atípicos possuem custo muito elevado se comparados aos típicos. Desta forma os antipsicóticos clássicos ou típicos continuam sendo largamente empregados no tratamento das psicoses. No entanto, o tratamento com estes fármacos possui eficácia comprometida por causar efeitos colaterais extrapiramidais agudos e crônicos como, por exemplo, a DT e o Parkinsonismo.

1.2.1 Flufenazina

A flufenazina (Figura 1) é um potente antipsicótico pertencente à classe das fenotiazinas, que foi introduzida na prática clínica no final da década de 50 (Darling, 1959; Taylor, 1959). Naquela época, a flufenazina foi considerada uma descoberta importante em relação às fenotiazinas já existentes (por exemplo, a clorpromazina) principalmente porque não produzia o efeito colateral da acatisia, comum a esta classe de medicamentos (Darling, 1959). Em 1957, Paul Janssen descobriu a atividade neuroléptica das butirofenonas. Nesta classe, encontra-se o haloperidol, um antipsicótico que se destaca por sua potência, especificidade e

longa ação (Niemegeers, 1983). A principal ação farmacológica dos antipsicóticos clássicos ou típicos (haloperidol e flufenazina) consiste em bloquear receptores dopaminérgicos D_2 (Creese et al., 1976).

Figura 1 – Estrutura química da flufenazina (Xu et al., 2014).



1.2.1.1 Farmacocinética da Flufenazina

A flufenazina está disponível comercialmente em formas de depósito esterificadas como enantato e decanoato (ambas dissolvidas em óleo de gergelim). Os antipsicóticos na forma depot são sintetizados por esterificação de seu grupo hidroxila com um ácido graxo de cadeia longa e dissolvidos em óleo de gergelim (no caso da flufenazina). Esta formulação, sendo administrada intramuscularmente, promove uma liberação prolongada do fármaco. A difusão e disponibilidade do fármaco livre liberado a partir do local de depósito do óleo é provavelmente o passo limitante inicial (Dreyfuss et al., 1976a) uma vez que a hidrólise enzimática a partir do éster ocorre rapidamente. O óleo de gergelim pode retardar a hidrólise resultando em uma duração de ação prolongada (Dreyfuss et al., 1976a; Jørgensen & Gollfries, 1972). Desta forma, a taxa de eliminação é aparentemente controlada pela taxa de absorção (liberação) e não pelo metabolismo hepático (Ereshefsky et al., 1984a; Jørgensen, 1980a). O tempo necessário para atingir concentrações plasmáticas estáveis é dependente da taxa de absorção do antipsicótico na forma de depósito e pode levar até 3 meses, enquanto que as concentrações plasmáticas são proporcionais a taxa de eliminação. O fármaco livre distribui-se através da barreira hemato-encefálica até o local de ação, podendo ser metabolizado a metabólitos ativos ou inativos, ou ligar-se a outros tecidos. Os metabólitos suficientemente polares formados são eliminados por via renal ou através da bile. O enantato de flufenazina atinge seu pico de concentração em 2 a 3 dias (Ereshefsky et al., 1984a).

A meia-vida de eliminação aparente do enantato de flufenazina é alcançada em 3,5-4 dias após uma única administração de 25 mg. Contudo, quando as concentrações de decanoato de flufenazina foram mensuradas em 4 pacientes mantidos em tratamento com administração semanal por mais de 5 semanas, a média de meia-vida aparente calculada foi 14,3± 2,2 dias.

(Whelpton & Curry, 1976). Este aumento na meia-vida deve-se provavelmente a redistribuição do fármaco ligado aos tecidos ou absorção residual do fármaco a partir dos sítios de administração. Não existem evidências da forma esterificada no plasma, urina ou fezes; contudo, as formas conjugadas da flufenazina, sulfóxido de flufenazina e 7-hidroxiflufenazina foram encontradas na urina. A flufenazina e a 7-hidroxiflufenazina foram encontradas nas fezes, mas não seus conjugados. O glicorunídio de 7-hidroxiflufenazina já foi encontrado na bile de cães e subsequentemente hidrolisado no trato gastrointestinal (Whelpton & Curry, 1976).

1.3. Discinesia Tardia

A DT consiste num distúrbio do movimento decorrente do uso prolongado de antipsicóticos, sendo considerada como o principal efeito colateral destes fármacos. As primeiras descrições desta síndrome foram publicadas entre 1956 e 1957. Inicialmente, a DT foi denominada de "discinesia persistente" sendo também referida como "síndrome buco-língüo-mastigatória" ou "síndrome da insuficiência extrapiramidal terminal" (Crane, 1968; Kane, 1995). O termo DT foi proposto em 1964, por Faurbye et al.

A DT caracteriza-se por movimentos anormais hipercinéticos, sem propósito, repetitivos e involuntários que podem ocorrer durante ou após a interrupção de um tratamento prolongado com antipsicóticos. Estes distúrbios do movimento ocorrem, mais freqüentemente, na região orofacial e incluem movimentos de mastigação, protrusão da língua, estalido dos lábios, movimentos de franzir a face e piscar os olhos (Andreassen & Jørgensen, 2000). Em alguns casos, os distúrbios hipercinéticos podem também atingir o pescoço, os membros (principalmente os superiores) e o tronco (Kane, 1995). Também podem desenvolver-se sintomas axiais de movimentos pélvicos para frente e para trás ou movimentos rotatórios, descontínuos, dos quadris. Estes sintomas possuem flutuações no decorrer do tempo, podendo variar em intensidade até mesmo dentro do mesmo dia (Gardos & Cole, 1983; Wolfarth & Ossowska, 1989; Laporta et al., 1990).

Alguns autores estimam que a média de prevalência da DT em pacientes recebendo tratamento com antipsicóticos clássicos seja em torno de 20–25% (Lee et al., 2014), aumentando com a idade. De fato, a DT atinge cerca de 50% dos pacientes, com mais de 50 anos de idade, em tratamento com antipsicóticos (Kane & Smith, 1982; Gardos et al., 1983; Yassa & Jeste, 1992). O mais sério aspecto da DT consiste na sua persistência por meses ou até anos após a retirada do tratamento, podendo ser irreversível (Crane, 1973; Jeste et al., 1979; Casey, 1985; Glazer et al., 1990).

1.4. MODELOS ANIMAIS DE DISCINESIA TARDIA

Nos modelos animais, a discinesia tardia é chamada de discinesia orofacial (DO). Dentre os modelos animais de DO destacam-se os modelos agudos induzidos por antipisicoticos e o modelo de DO induzido por reserpina por serem os mais comumente utilizados. Contudo, esses modelos agudos têm sido criticados devido a uma série de fatores. A principal crítica consiste no fato de a síndrome extrapiramidal aguda apresentar mais similaridades com Parkinsonismo do que com a DT propriamente dita (Egan et al., 1996). Além disso, sabe-se que, em humanos, a retirada do tratamento prolongado com antipsicóticos leva a uma exacerbação da síndrome, o que é visto apenas em modelos crônicos de DO (Gunne et al., 1982; Egan et al., 1994). Os fármacos comumente utilizados como indutores de DO crônica são o haloperidol e a flufenazina sendo que o tratamento com flufenazina leva a uma prevalência de 70-80% enquanto que para o haloperidol estes valores são de 50% (Fachinetto et al., 2007a; Fachinetto et al., 2007b; Peroza et al., 2013).

Algumas similaridades entre a DO em animais e DT em humanos podem ser observadas. Tanto em humanos como em animais existe um subgrupo que é mais suscetível ao desenvolvimento da síndrome (Tamminga et al., 1990; Egan et al., 1994; Shirakawa & Tamminga, 1994). Os movimentos orofaciais induzidos por antipsicóticos em animais possuem a mesma freqüência (1-3 Hz) da DT em humanos (See & Ellison, 1990). Como em humanos, o número de movimentos de mascar no vazio (MMV), que consiste no parâmetro mais utilizado para avaliação do desenvolvimento da DO, possui flutuações no decorrer do tempo e pode piorar muito quando existe um fator de estresse envolvido (Waddington, 1990; Kaneda et al., 1992; Egan et al., 1994). Em animais também é visto um aumento da DO com o aumento da idade tanto induzida por antipsicóticos quanto espontânea (Kaneda et al., 1992; Egan et al., 1994; Jørgensen et al., 1994; Andreassen et al., 1996; 1998). De uma maneira geral, tanto em humanos como em modelos animais, o sistema dopaminérgico desempenha um importante papel na gênese da DT e DO.

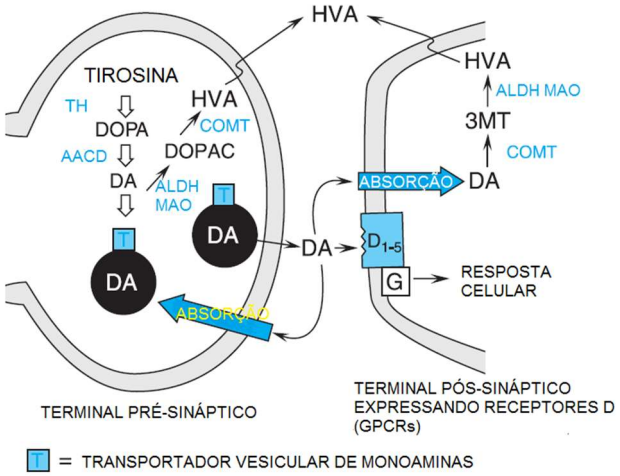
1.5 SISTEMA DOPAMINÉRGICO

Existem quatro vias dopaminérgicas principais no cérebro de mamíferos, as quais compõem o sistema dopaminérgico: via mesolímbica, via mesocortical, via tuberoinfundibular e via nigroestriatal (Dahlstrom & Fuxe, 1964). A via nigroestriatal, que contém cerca de 80% da dopamina cerebral, tem seus corpos celulares na *substantia nigra* e suas projeções para o corpo estriado (Albin et al., 1989). Esta via está envolvida no funcionamento normal dos gânglios da base e a degeneração dos neurônios dopaminérgicos nigroestriatais leva aos

sintomas motores que caracterizam a Doença de Parkinson e a DT por ser uma via que exerce controle inibitório sobre outras vias não dopaminérgicas (Crossman, 1990; 2000).

O sistema dopaminérgico possui como neurotransmissor a dopamina, cuja síntese inicia-se a partir do aminoácido tirosina e tem como etapa limitante a conversão da tirosina a DOPA pela enzima tirosina hidroxilase (TH). A DOPA é convertida em dopamina pela enzima DOPA descarboxilase. A dopamina é, então, transportada do citoplasma onde é sintetizada para vesículas de estocagem especializadas, via transportador vesicular de monoaminas 2 (TVMA-2). Em resposta ao estímulo do neurônio pré-sináptico ocorre a abertura de canais de cálcio dependentes de voltagem (Goodman & Gilman, 2010). O aumento na concentração de cálcio citosólico leva a fusão das vesículas de dopamina com a membrana plasmática e a liberação deste neurotransmissor na fenda sináptica. A dopamina presente no espaço sináptico pode agir em receptores dopaminérgicos exercendo suas ações celulares. Para a redução dos níveis de dopamina extracelular, a dopamina presente no espaço sináptico pode ser transportada para o interior dos neurônios pré-sinápticos pelo transportador de dopamina (TDA) (Amara & Kuhar, 1993). Além disso, a ligação da dopamina em receptores pré-sinápticos D_2 inibe sua síntese diminuindo, desta forma, seu armazenamento e liberação (Albin et al., 1989). Os principais metabólitos da dopamina formados no sistema nervoso central (SNC) são principalmente o ácido homovanílico (HVA) e o ácido 3,4-diidroxifenilacético (DOPAC), sendo estes formados principalmente através da atividade da enzima monoaminaoxidase (MAO) acoplada na membrana mitocondrial e da catecol-O-metiltransferase (COMT) (Figura 2).

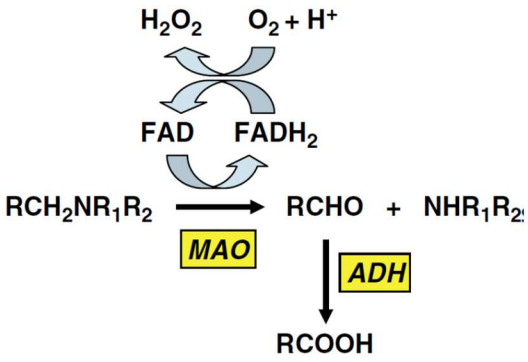
Figura 2: Síntese e metabolismo da dopamina: TH – tirosina hidroxilase; DOPAC – ácido3,4-diidroxifenilacético; DA – dopamina; COMT – catecol-O-metiltransferase; HVA – ácido homovanílico; AACD – L-aminoacido aromático descarboxilase; 3MT – 3-metoxitiramina; ALDH – aldeído desidrogenase (Goodman & Gilman, 2010)



1.5.1 Monoaminoxidase

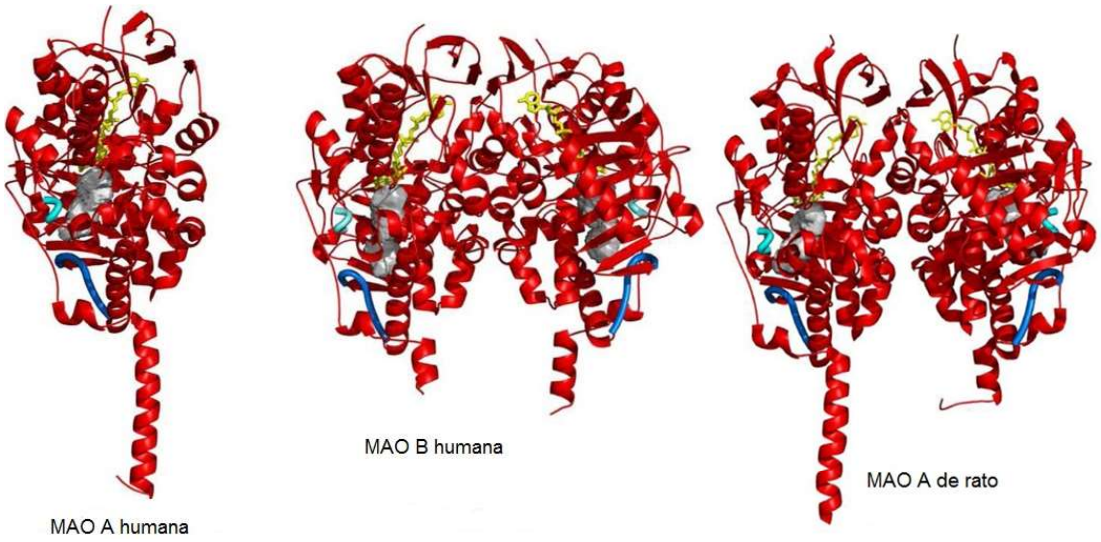
A enzima monoaminoxidase (MAO) é responsável pela desaminação oxidativa de diversas aminas biogênicas, incluindo os neurotrasmissores serotonina, noradrenalina e dopamina. A reação da MAO envolve a desaminação oxidativa de aminas primárias, secundárias e terciárias a seus aldeídos e amina livre correspondentes, com a geração de peróxido de hidrogênio (Figura 3). O aldeído é rapidamente metabolizado pela enzima aldeído desidrogenase em metabólitos acídicos. Estes metabólitos (ácido 5-hidroxiindol acético (5-HIAA) da 5-hidroxitriptamina (5-HT, serotonina) ou ácido diidroxi-fenilacético (DOPAC) da dopamina) são comumente usados para mensurar a atividade da MAO *in vitro* ou *in vivo* (Youdim & Blakhle 2006).

Figura 3: Reação de desaminação oxidativa de aminas biogênicas pela MAO mitocondrial: H_2O_2 – peróxido de hidrogênio; O_2 – oxigênio; H^+ - radical hidroxil; FAD – flavina adenina dinucleotideo; $FADH_2$ – flavina adenina dinucleotídeo (forma reduzida); $HCH_2NR_1R_2$ – amína; MAO – monoaminoxidase; RCHO – aldeído; NHR_1R_2 – amina livre; ADH – aldeídodesidrogenase; RCOOH metabólito ácido (Youdim & Blakhle 2006).



Com base em estudos farmacológicos, bioquímicos e genéticos, duas isoformas da MAO foram identificadas e denominadas como MAO-A e MAO-B com cerca de 70% de homologia (Bach et al., 1988; Johnston, 1968; Magyar & Knoll, 1977). As estruturas tridimensionais da MAO-B humana (Binda et al., 2002), MAO-A humana (De Colibus et al., 2005; Son et al., 2008), e MAO-A de rato (Ma et al., 2004) estão ilustradas na Figura 4.

Figura 4: Estruturas tridimensionais da MAO-A humana, MAO-B humana e MAO-A de rato (Edmondson et al., 2009).



Ambas MAO-A de rato e MAO-B humana cristalizam como dímeros com interação monômero-monômero similares. A MAO-A humana difere por cristalizar como um monômero. Isto reflete uma diferença no amino ácido da posição 151 onde a MAO-A de rato possui um resíduo de ácido glutâmico enquanto que a MAO-A humana possui uma lisina. A região C-terminal da MAO-B humana e da MAO-A de rato são α-helices transmembrana que ancoram a enzima à membrana mitocondrial externa, com o resto da proteína exposta ao citoplasma (Binda et al., 2008). A entrada do inibidor ou do substrato no sítio ativo da MAO-B humana ou da MAO-A de rato parece ocorrer próximo da intersecção com a superfície da membrana. Uma das principais diferenças entre a MAO-A de rato e a MAO-B humana é a forma e configuração de seus respectivos locais de ligação ao substrato. Uma diferença significativa na estrutura da MAO-A humana e de rato é a conformação da alça que forma a cavidade nos resíduos 210–216 (Binda et al., 2008). Na MAO-A de rato esta alça é o contrário do que a encontrada na MAO-A humana, embora homologa a MAO-B humana. Esta diferença é responsável pelo menor volume da cavidade do sitío ativo da MAO-A de rato (Youdim et al., 2006). Para a maioria das espécies de mamíferos, a MAO-A preferencialmente desamina a serotonina e é seletivamente inibida por baixas concentrações de selegilina. A dopamina, a noradrenalina e a tiramina são oxidadas por ambas as isoformas na maioria das espécies (Youdim et al., 2006). Contudo existem diversas exceções, pois a expecificidade da MAO por seu substrato depende da concentração, da afinidade e da taxa de renovação do substrato, assim como da concentração da enzima e da espécie analisada (Shih et al., 1999).

A MAO é expressa na maioria dos tecidos de mamíferos, contudo a proporção das duas isoformas varia entre os tecidos. Além disso, a distribuição da MAO-A e da MAO-B no SNC exibe pouca variação entre as espécies (Shih et al., 1999). No tecido cerebral, a MAO-A se encontra predominantemente em regiões com alta densidade de neurônios catecolaminérgicos como *lócus coeruleus*, *substantia nigra* e regiões periventriculares do hipotálamo. Em contraste, a MAO-B é preferencialmente expressa em neurônios serotoninérgicos (células do núcleo dorsal da *rafe*) e em astrócitos (Jahng et al., 1997; Saura et al., 1996; Westlund et al., 1985). Tem sido sugerido que a MAO-A e a MAO-B neuronais possuem um papel importante em proteger os neurônios de aminas exógenas, em terminar as ações das aminas neurotransmissoras, assim como em regular os estoques de aminas intercelulares (Youdim et al., 2006). Em tecidos periféricos, como intestino, fígado, pulmão e placenta, a MAO-A protege o organismo oxidando aminas provenientes do sangue ou prevenindo sua entrada na circulação sanguínea. Em microvasos da barreira hemato-encefálica, por exemplo, a MAO possui função protetora agindo como uma barreira metabólica (Youdim et al., 2006). Além disso, a inibição da MAO-B é muito explorada para fármacos com ação antiparkinsoniana (Marconi & Zwingers, 2014), visto que a inibição da MAO-A causa efeitos antiapoptóticos (Ou et al., 2006). Devido à sua função no metabolismo das catecolaminas, a MAO parece exercer um papel importante na patofisiologia de diversos distúrbios neurológicos e psiquiátricos.

1.6. HIPÓTESES PARA A DISCINESIA TARDIA

Algumas hipóteses têm sido propostas na tentativa de elucidar o mecanismo de desenvolvimento da DT. Embora sua exata patofisiologia permaneça não esclarecida, alterações no sistema dopaminérgico vêm sendo amplamente descritas por terem papel crucial no desenvolvimento da DT. A seguir está descrito um apanhado geral sobre algumas das hipóteses que tentam explicar a gênese da DT.

1.6.1. Hipótese da supersensibilidade dopaminérgica

A supersensibilidade dopaminérgica consiste na mais popular hipótese para explicar o desenvolvimento da DT após uso crônico de neurolépticos. Segundo esta hipótese, a DT é resultante de uma resposta do SNC, secundária ao bloqueio crônico dos receptores dopaminérgicos pelos antipsicóticos, em locais relacionados ao controle dos movimentos. Em resposta a este bloqueio crônico, há um aumento compensatório no número de receptores dopaminérgicos, os quais provavelmente por responderem a menores níveis de DA, levam a um estado hiperdopaminérgico e a manifestações clínicas como, por exemplo, a DT (Klawans

& Rubovits, 1972; Burt et al., 1977; Rubinstein et al., 1990). No entanto, esta hipótese possui algumas contradições. Sua principal inconsistência é que o mais importante fator de risco para o desenvolvimento da DT é a idade (Cavallero & Smeraldi, 1995; Kane, 1995; Woerner et al., 1998). Contudo, foi demonstrado que o envelhecimento faz com que ocorra a redução tanto do número quanto da sensibilidade dos receptores dopaminérgicos (Lohr & Jeste, 1988; Sachdev, 1999).

Apesar da hipótese da supersensibilidade dos receptores dopaminérgicos possuir algumas inconsistências, o sistema dopaminérgico parece estar diretamente relacionado ao desenvolvimento da DT e DO.

1.6.2. Hipótese dos radicais livres

Outra hipótese sugere que os radicais livres possam ter uma importante participação no desenvolvimento da DT (Cadet et al., 1987; Lohr et al., 2003). A hipótese dos radicais livres tem como base para o seu desenvolvimento o sistema dopaminérgico. De acordo com isto, dados da literatura demonstram que a administração de antipsicóticos, por bloquear receptores dopaminérgicos pré-sinápticos responsáveis pela retroinibição da síntese de dopamina (DA), levam a um aumento secundário de sua síntese e, elevação nos níveis extracelulares deste neurotransmissor e, consequentemente, a um aumento no seu metabolismo via aumento da atividade da MAO (Lohr, 1991; Andreasen & Jørgensen, 2000). Sabe-se que a atividade das oxidases em geral, forma como produto o peróxido de hidrogênio (H_2O_2) que ao reagir com metais de transição via reação de fenton, forma radicais livres, como os radicais hidroxila ($OH^{\cdot}$) e o radical ânion superóxido. Além disso, a própria dopamina pode sofrer auto-oxidação formando quinona de dopamina que age como uma espécie reativa de oxigênio (Lohr, 1991; 2003). Concordando com o fato de os radicais livres estarem envolvidos no desenvolvimento da DO, foi demonstrado que a administração de substâncias pró-oxidantes é capaz de potencializar o desenvolvimento de DO em modelos animais (Andreassen et al., 1998; Calvent et al., 2002; Fachinetto et al., 2005).

Sabe-se que o cérebro é particularmente vulnerável à ação tóxica das espécies reativas de oxigênio (EROS), devido a alta produção vinculada à grande quantidade de energia utilizada pelo tecido via metabolismo oxidativo (Lohr, 1991). Além disso, o SNC, é extremamente rico em ácidos graxos poliinsaturados, característica que propicia a peroxidação lipídica (Lohr et al., 2003). Dados da literatura têm demonstrado que, em animais tratados com antipsicóticos, existe aumento nos níveis de peroxidação lipídica e de carbonilação de proteínas, redução na atividade de enzimas antioxidantes como a superóxido dismutase, a catalase e a glutationa

peroxidase, e também redução da glutationa reduzida e conseqüente aumento da glutationa oxidada (Post et al., 2002; Naidu et al., 2003a; Abílio et al., 2004; Burger et al., 2004; 2005a; 2005b; Faria et al., 2005; Sadan et al., 2005; Pillai et al., 2007). De acordo com tais constatações, existem evidências que o uso de substâncias com potencial antioxidante atenua ou mesmo reverte a DO (Sachdev et al., 1999; Naidu et al., 2003a; 2003b; Burger et al., 2004; 2005a; 2006; Fachinetto et al., 2007b; Busanello et al., 2011; Reckziegel et al., 2013; Peroza et al., 2013).

Em humanos foi demonstrado que existe aumento de parâmetros oxidativos no fluído cérebro espinhal e plasma de pacientes com DT (Pall et al., 1987; Tsai et al., 1998; Lohr et al., 1990). Foi relatado que o uso de substâncias antioxidantes como a vitamina E pode prevenir o aparecimento da discinesia em pacientes (Egan et al., 1992; Dabiri et al., 1994). No entanto, seu uso como tratamento após a síndrome já instalada, parece não ter eficácia.

Tendo em vista que o metabolismo da dopamina pode levar a um aumento das EROS, diversos trabalhos têm investigado o papel de antioxidantes em modelos de distúrbios motores com efeito promissor em alguns casos. Neste contexto, o resveratrol pode ser considerado promissor no tratamento de desordens motoras relacionadas ao sistema dopaminérgico uma vez que além da ação antioxidante, possui algumas ações protetoras relacionadas ao sistema dopaminérgico.

1.7. RESVERATROL

O resveratrol (trans-3,5,4'-trihidroxiistilbeno) é um polifenol presente em altas concentrações na casca e sementes de uva e no vinho tinto, sendo primeiramente conhecido por ser o componente principal do Ko-Jo-Kon, extraído das raízes do *Poligonum cuspidatum* (Nonomura et al., 1963). Este vegetal é cultivado comercialmente na China para a produção do resveratrol usado em suplemento dietético. No Japão, este vegetal é utilizado no preparo do chá de Itadori, que representa a fonte não alcoólica do resveratrol (Delmas et al., 2005).

Na videira, o resveratrol é produzido em resposta à agressão de fungos e concentra-se nas células da película e sementes das uvas, sendo seu teor maior no vinho tinto (Frémont, 2000; Gehm et al., 1997). O vinho tinto contém maior quantidade de resveratrol do que os outros vinhos, por causa do processo de fabricação. Na elaboração do vinho tinto as uvas vermelhas são colocadas para fermentar com a casca, sementes e talos, enquanto, na fabricação dos outros vinhos, utiliza-se o suco das uvas obtido por pressão (Oak et al., 2005). Os níveis de resveratrol encontrados nos vinhos tintos variam muito, situando-se em média entre 0,82 e 5,75 mg/L podendo chegar a 9 mg/L, enquanto o suco de uva comercial contém aproximadamente 0,07 a

1,59 mg/L de resveratrol (Souto et al., 2001; Dong, 2003). Souto et al. (2001), verificaram que a média de resveratrol nos vinhos brasileiros fica em torno de 3,57 mg/L, sendo uma das mais altas do mundo. Esses valores, provavelmente devem ser resultantes da alta precipitação pluviométrica na região da Serra Gaúcha, o que favorece a proliferação de doenças fúngicas na parte aérea das videiras (Souto et al., 2001; Machado et al., 2011).

1.7.1. Farmacocinética do resveratrol

O resveratrol apresenta propriedades lipofílicas e atravessa a membrana plasmática das células sendo absorvido quando administrado por via oral. É metabolizado no organismo e pode interagir com e modular enzimas de fase I como CYP1A2, CYP3A4 e CYP2D6 e de fase II, glutationa-S-transferase (GST) e catecol-O-metiltransferase (COMT) (De Santi et al., 2000a; 2000b). Estudos farmacocinéticos em seres humanos e a partir da extrapolação de linhagens de célula humana sugerem que 25 mg de resveratrol administrados por via oral são absorvidos significativamente por meio de difusão trans-epitelial. O resveratrol possui uma meia vida de cerca de 9 horas e o pico de concentração plasmática do metabólito ativo é de aproximadamente 2 μM (Wen & Walle, 2006; Walle et al., 2004). O alto e extenso metabolismo de resveratrol no intestino e fígado resulta em aproximadamente 1% de biodisponibilidade do composto original (Walle et al., 2004; Walle, 2011). Curiosamente, a biodisponibilidade do resveratrol é relatada ser maior durante a manhã devido ao ciclo circadiano, uma consideração importante para determinar os horários de administração (Waterhouse, 2009; Almeida et al.; 2009). Os metabólitos e polímeros do resveratrol permanecem no plasma muito mais tempo do que o resveratrol não convertido, ao passo que o resveratrol metilado permanece na corrente sanguínea por um período ainda mais longo, propriedade essa que tem sido explorada no desenvolvimento de drogas análogas ao resveratrol (Pervaiz & Holme, 2009).

Os estudos *in vivo* indicam que o resveratrol é absorvido e distribuído para vários tecidos (fígado, rim, coração e cérebro) a partir do plasma, sendo estes processos dependentes do tempo e da concentração de exposição (Walle, 2011; Andres-Lacueva et al., 2012; Clark et al., 2012). O resveratrol pode também ser rapidamente conjugado na forma de monossulfato e dissulfato e pode ser completamente metabolizado dentro de 8 horas em hepatócitos humanos e células HepG2 (Lancon et al., 2004; 2007). Alguns dos metabólitos mais abundantes do resveratrol em mamíferos são o resveratrol-3-sulfato, o resveratrol-3-O-glucuronídeo, e o dihidro-resveratrol; no entanto, eles não estão totalmente caracterizados (Walle et al., 2004). Alguns outros metabólitos foram identificados e caracterizados (Schwedhelm et al., 2003). Estudos *in vitro* indicam que 50-98% do resveratrol total se liga de forma não covalente à albumina, lipoproteína

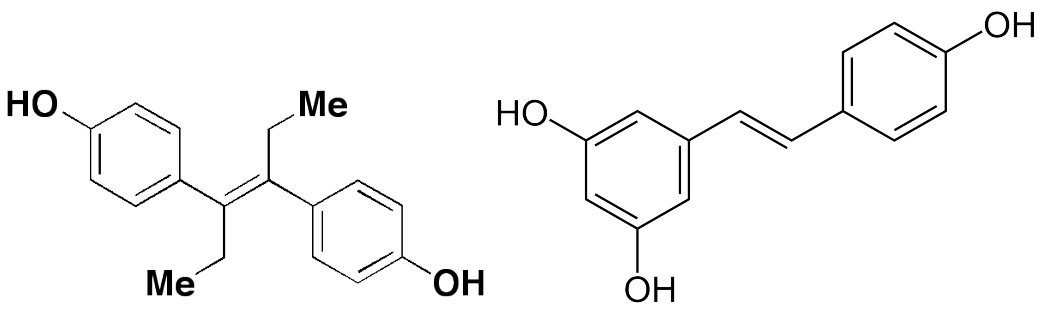
de baixa densidade, e hemoglobina (Jannin et al., 2004; Lu et al., 2007). Em seres humanos, cerca de 50% dos metabólitos do resveratrol são transportados no plasma ligado às proteínas (Burkon & Somoza, 2008). Os rins são a via de excreção dominante com recuperação urinária e fecal do resveratrol total entre 98% e 70% em 24 h (Boocock et al., 2007a; 2007b).

Em um estudo em humanos, com 40 indivíduos saudáveis, foram testadas de 1 a 29 doses repetidas, e os resultados indicaram que o resveratrol foi bastante tolerável e causou efeitos colaterais leves de náuseas e dor de cabeça (Brown et al., 2010; Cottart et al., 2010). Ocasionalmente, em doses mais elevadas foi também relatado diarréia em comparação com placebo (Brown et al., 2010; Patel et al., 2010). Assim, devido o resveratrol ser um polifenol de baixa biodispnibilidade sendo transformado rapidamente em seus metabolitos *in vivo*, é provável que algumas das atividades encontradas sejam referentes a esses metabólitos e não ao seu estado nativo (Singh et al., 2013).

1.7.2 Propriedades do reveratrol

O resveratrol é utilizado há muito tempo na terapêutica medicinal Oriental, sendo usado pelos chineses e japoneses para o tratamento de arteriosclerose, de doenças inflamatórias e alérgicas. Suas características polifenólicas permitem explicar suas atividades como anti-agregante plaquetário, antioxidante e redutora de triglicerídeos (Belguendouz et al., 1998; Meyer et al., 1997). De fato, o resveratrol diminui os níveis de lipídeos no soro sanguíneo, aumenta o colesterol HDL, que ajuda a remover o colesterol LDL do sangue e a prevenir a obstrução das artérias (Galfi et al., 2005). Sua estrutura molecular é similar à estrutura do estrogênio sintético, o dietilestilbestrol (Figura 5). Portanto, tem propriedades farmacológicas similares à do estradiol, principal estrogênio humano natural (Bradamante et al., 2004).

Figura 5 – Estruturas moleculares do dietilestilbestrol e do trans –resveratrol (Bradamante et al., 2004).



De particular importância, dados da literatura têm demonstrado que o resveratrol possui ação protetora contra a neurotoxicidade dopaminérgica em animais experimentais (Jin et al., 2008; Blanchet et al., 2008), incluindo desordens motoras induzidas por reserpina (Busanello et al., 2011), além de inibir a MAO sendo sugerido que ele possua ação antidepressiva (Hurley et al., 2014). Um estudo demonstrou que o resveratrol foi capaz de restaurar a expressão do TDA em neurônios dopaminérgicos tratados com 1-metil-4-fenil-piridínio (MPP^+) (Blanchet et al., 2008; Gélinas & Martinoli, 2002). Além disso, dados da literatura demonstraram que o resveratrol, através da interação com receptores de estrógeno, aumenta a expressão do TDA em cultura de neurônios dopaminérgicos humanos e em estriado de fêmeas de camundongos (Di Liberto et al., 2012). No entanto, não existem relatos na literatura demonstrando se o reveratrol exerce efeito na DO induzida por antipsicóticos e se seu mecanismo de ação pode envolver a atividade da MAO.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o efeito do resveratrol sobre as alterações comportamentais induzidas por flufenazina em ratos e sua possível relação com a atividade da enzima MAO.

2.2 OBJETIVOS ESPECÍFICOS

→ Avaliar o efeito do resveratrol em modelo agudo de discinesia orofacial induzida por flufenazina em ratos;

→ Determinar *in silico* e *in vitro* o efeito do resveratrol sobre a enzima MAO;

→ Investigar o efeito do resveratrol sobre parâmetros comportamentais e atividade da MAO em ratos tratados cronicamente com flufenazina.

3. RESULTADOS

3.1 ARTIGO

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Título: Resveratrol reduces vacuous chewing movements induced by acute treatment with fluphenazine

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Resveratrol reduces vacuous chewing movements induced by acute treatment with fluphenazine

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ABSTRACT

Treatment with classical neuroleptics in humans can produce a serious side effect, known as tardive dyskinesia (TD). Here, we examined the possible neuroprotective effects of resveratrol, a polyphenol compound contained in red grapes and red wine, in an animal model of orofacial dyskinesia (OD) induced by acute treatment with fluphenazine. Adult male rats were treated during 3 weeks with fluphenazine enantate (25 mg/kg, i.m., single administration) and/or resveratrol (1 mg/kg, s.c., 3 times a week). Vacuous chewing movements (VCMs), locomotor and exploratory performance were evaluated. Fluphenazine treatment produced VCM in 70% of rats and the concomitant treatment with resveratrol decreased the prevalence to 30%, but did not modify the intensity of VCMs. Furthermore, the fluphenazine administration reduced the locomotor and exploratory activity of animals in the open field test. Resveratrol co-treatment was able to protect the reduction of both parameters. Taken together, our data suggest that resveratrol could be considered a potential neuroprotective agent by reducing motor disorders induced by fluphenazine treatment.

1. Introduction

Schizophrenia is a chronic and debilitating psychiatric illness affecting millions of people worldwide, with a median prevalence of 1%. The most efficacious treatment includes the use of neuroleptic drugs, whose action mechanism consists mainly in the blockade of D_2 receptors in the central nervous system (Creese et al., 1976). However, the chronic treatment with neuroleptics produces a motor disorder that is characterized by involuntary movements of the orofacial region and, sometimes, musculature of the members and trunk, known as tardive dyskinesia (TD) (Kane, 1995; Kane and Smith, 1982; Woerner et al., 1991). This syndrome presents a high prevalence in humans taking neuroleptic drugs and can persist for months or years after drug withdrawal, and in some patients it is irreversible (Casey, 1985; Crane, 1973; Jeste et al., 1979). The molecular mechanisms responsible for TD are still not completely understood. However, several hypothesis reinforced by experimental data have been postulated to explain the neuropathophysiology of TD (Andreassen and Jorgensen, 2000; Ebadi and Srinivasan, 1995; Lohr et al., 2003). Among them are the classical hypothesis of the occurrence of the supersensitivity of dopamine receptors; the disturbed balance between dopaminergic and cholinergic systems; the dysfunction of striatonigral GABAergic neurons; excitotoxicity and, more recently the hypothesis of free radicals (Andreassen and Jorgensen, 2000; Cadet et al., 1986, 1987; Ebadi and Srinivasan, 1995; Lohr et al., 1987, 2003). Although all these evidences point to common event of neurotoxicity in brain areas involved in modulation of movement; the precise molecular mechanisms that could promote the development and maintenance of TD is still not elucidated.

The recognized model of orofacial dyskinesia (OD) in animals, using the number of vacuous chewing movements (VCMs) as main parameter of analysis, has been extensively investigated by several groups in the search for promissory agents that could reduce the prevalence and/or intensity of OD; and consequently offers beneficial effect against the motor alterations associated to the use of neuroleptics (Andreassen et al., 2003; Abílio et al., 2004; Burger et al., 2004, 2005a; 2005b; Fachinetto et al., 2005, 2007a, 2007b; Faria et al., 2005; Naidu et al., 2003; Post et al., 2002; Sadan et al., 2005).

In this view, resveratrol, a polyphenol primarily contained in red grapes and red wine, is known to exert several pharmacological properties including antioxidant, anticancer and neuroprotective activities in different *in vitro* and *in vivo* experimental models (Anekonda, 2006; Brown et al., 2009; Calabrese et al., 2008; Gélinas and Martinoli, 2002; Hall et al., 2010; Okawara et al., 2007). In accordance, recent studies performed in humans and animals have indicate that

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moderate consumption of red wine reduces the incidence of dementia and Alzheimer's disease (Ho et al., 2009; Vingtdeux et al., 2008). Besides, resveratrol shows no overt signals of toxicity in doses commonly used in humans (Cottart et al., 2010). However, although many studies demonstrate the potent neuroprotective effect of resveratrol, is currently unknown the role of resveratrol against OD induced by utilization of neuroleptics. Thus, this work examines, for the first time, the possible protective effect of a low dose resveratrol in an acute VCM model induced by fluphenazine in rats. Thereby, we evaluated the effect of both treatments on locomotor activity, in order to determinate if fluphenazine and/or resveratrol would change spontaneous and exploratory behavior of animals.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 270–320 g, from our own breeding colony were kept in cages with continuous access to food and water in a room with controlled temperature (22 ± 3 °C) and on a 12-h light/dark cycle with lights on at 7:00 am. The animals were maintained and used in accordance to the guidelines of the Brazilian Society of Association for Laboratory Animal Science. This protocol was submitted and approved by Ethical intern commission of UFSM sob number 051/2011.

2.2. Drugs and treatments

Fluphenazine enantate (Flufenan®) and resveratrol (3,4,5-trihydroxy-trans-stilbene) were obtained from commercial pharmacy. Rats were randomly divided into the following groups: (1) control (n = 5), (2) resveratrol (n = 5), (3) fluphenazine (n = 10) and (4) fluphenazine plus resveratrol (n = 10). Fluphenazine groups received a single intramuscular (i.m.) injection of fluphenazine enantate (25 mg/Kg), which was at first day of experimental period (Van Kampen and Stoessl, 2000) and its vehicle consisted of soy oil administered 1 mL/kg, i.m. The treatment with resveratrol started concomitantly to fluphenazine injection. Resveratrol was administered subcutaneously (s.c.) 3 times per week (in non-consecutive days) at the dose of 1 mg/kg for 21 days. Resveratrol was dissolved in soy oil and the control rats were similarly treated with the vehicle (1 mL/kg).

2.3. Behavioral analysis

2.3.1. Quantification of VCMs

Behavior was assessed before the treatment with vehicles, fluphenazine and/or resveratrol. The effect of drugs on behavior was examined 21 days after the fluphenazine injection. To quantify the occurrence of OD, rats were placed individually in cages (20 × 20 × 19 cm) and hand operated counters were employed to quantify VCM frequency. VCMs are defined as single mouth openings in the vertical plane not directed towards physical material. VCM parameters were not scored during grooming or rearing periods. VCMs were measured continuously for 6 min after a period of 6 min adaptation. During the observation sessions, a mirror was placed under the floor of the experimental cage to permit observation when the animal was faced away from the observer. Experimenters were always blind to the treatments given to rats. It was previously reported that the treatment with neuroleptic drugs does not result in the development of OD in all treated rats (Kane and Smith, 1982; Shirakawa and Tamminga, 1994). Thus, in the present study, we have verified the prevalence and intensity of neuroleptic-induced VCMs as previously described (Fachinetto et al., 2007a, 2007b) as well as the effect of resveratrol on this parameter.

2.3.2. Open field test

To analyze changes in spontaneous locomotor and exploratory activities caused by treatment with fluphenazine and/or resveratrol, the animals were placed individually in the center of a circular open-field arena divided into 9 parts, as previously described (Broadhurst, 1960). The number of line crossings and number of rearing were measured over 5 min. This experiment was performed immediately before VCMs quantification at 21th day of treatment.

2.4. Statistical analysis

Data from orofacial dyskinesia (VCM) and open field tests (rearing and crossings) were analyzed by one-way ANOVA, followed by Tukey's Post Hoc tests when appropriate. F values are presented in the text only if p value associated with it was <0.05. Prevalence data were analyzed by the Chi-square test. Significance was considered when $p<0.05$.

3. Results

3.1. Effects of resveratrol on VCM induced by acute treatment with fluphenazine in rats

Statistical analysis revealed that fluphenazine administration caused a marked increase on VCM intensity when compared with its vehicle ($F(5,29) = 20.51$ and $p<0.001$; Fig. 1). The co-treatment with resveratrol protected the increase in VCM intensity (Fig. 1).

After observing that resveratrol reduced the frequency of VCM, we investigated the prevalence of VCM in these animals and the VCM intensity separately in those that developed and did not develop VCM, since not all animals develop VCM after administration of neuroleptics.

Fluphenazine induced a high prevalence of VCMs in rats, with 70% of treated rats presenting more than 40 VCM when compared with its respective vehicle (Chi-square = 6.56 and $p<0.05$; Table 1). Interestingly, resveratrol treatment was able to reduce the prevalence of VCMs to 30% (Chi-square = 3.2 and $p = 0.07$) in fluphenazine-administered rats (Table 1). However, this effect did not reach significantly. On the order hand, resveratrol was not effective in modify the number of VCMs in those animals that developed VCMs (Table 1). Furthermore, the administration of resveratrol alone did not cause any alteration in animal behavior (Fig. 1 and Table 1).

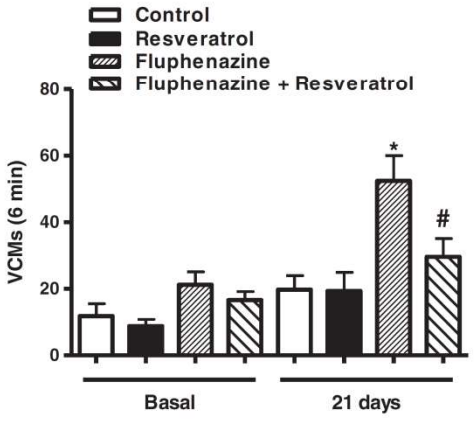

Fig. 1. Effect of resveratrol on VCM intensity induced by fluphenazine in rats. One-way ANOVA followed by Tukey's multiple range tests. * represent significant difference from control group. # represent significant difference from fluphenazine group.

Table 1
Effect of resveratrol on VCM intensity and prevalence induced by fluphenazine in rats. Values are presented as means ± S.E.M.

Groups	VCM intensity	VCM prevalence
Control	19.80 ± 4.14	0/5
Resveratrol	19.40 ± 5.55	0/5
Fluphenazine (−VCM)	24.00 ± 1.73	3/10
Fluphenazine (+VCM)	64.57 ± 6.45[a]	7/10
Fluphenazine + resveratrol (−VCM)	19.71 ± 3.21	7/10
Fluphenazine + resveratrol (+VCM)	52.67 ± 1.20[a]	3/10

One way ANOVA followed by Tukey's multiple range tests.
[a] Represent significant difference from control, fluphenazine and fluphenazine + resveratrol groups.

3.2. Effects of acute treatment with fluphenazine and/or resveratrol on locomotor and exploratory activity in rats

Fluphenazine administration caused a significant decrease on locomotor activity ($F(3,29) = 3.22$ and $p<0.05$), represented by the number of crossings in the open field test (Table 2). Resveratrol treatment was not able to prevent the reduction in locomotor activity caused by fluphenazine to the control levels (Table 2). Moreover, crossing number was not modified in the group treated with resveratrol alone.

Similarly, fluphenazine caused a marked reduction in the exploratory activity ($F(3,29) = 3.54$ and $p<0.05$), represented by the number of rearing in the open field test, that was not prevented by resveratrol treatment. As verified with crossing number, resveratrol alone also did not change rearing behavior.

4. Discussion

TD and OD occurrences have been a major problem associated with chronic neuroleptic treatment in humans and animals models respectively. In accordance, in the current study we demonstrated that an acute administration of fluphenazine produced OD in the animals. Although the precise mechanisms that contribute to the development of OD remain elusive; several research have indicate the involvement of multiple neurotransmitter systems and receptor types in this motor disorder (see for review Andreassen and Jorgensen, 2000; Gunne et al., 1984; Lee et al., 1997; Lohr et al., 2003). In fact, dopamine receptors supersensitivity, disturbed balance between dopaminergic and cholinergic systems, dysfunction of striatonigral GABAergic neurons and excitotoxicity involving glutamatergic systems are generally suggested as possible events associated to neuropathophysiology of OD and TD (Andreassen and Jorgensen, 2000; Cadet et al., 1987; Burger et al., 2005b; Lohr et al., 2003;). Indeed, one other hypothesis that has pointed to a possible mechanism related to development of OD and TD is the participation of oxidative stress (Andreassen and Jorgensen, 2000; Lohr et al., 2003). In contrast, our group recently reported that OD induced by chronic treatment with both neuroleptics haloperidol and fluphenazine was not directly associated with changes in oxidative stress endpoint parameters quantified in our studies (Fachinetto et al., 2007a, 2007b).

Table 2
Effect of resveratrol on open field test in rats fluphenazine treated. Values of number of crossings and rearing are presented as means ± S.E.M.

	Control	Resveratrol	Fluphenazine	Fluphenazine + resveratrol
Crossings	48.67 ± 6.69[a]	36.00 ± 6.35[a]	15.90 ± 4.51[b]	22.44 ± 3.46[a,b]
Rearings	17.33 ± 0.33[a]	12.40 ± 2.11[a]	3.70 ± 1.04[b]	8.60 ± 3.38[a,b]

Different markers mean statistical differences among the groups. One way ANOVA followed by Tukey's multiple range tests. (Control, n = 5; resveratrol, n = 5; fluphenazine, n = 10; fluphenazine + resveratrol, n = 10).

Instead, we have found a decrease in dopamine uptake which was related to VCM development (Fachinetto et al., 2007a, 2007b).

Resveratrol is a polyphenol primarily contained in red grapes and red wine and it is quickly absorbed in the organism. It has been demonstrated that resveratrol crosses the blood–brain barrier and is incorporated in the brain tissues (Wang et al., 2002). Moreover, due to its high lipid solubility, trans-resveratrol might be deposited in adipose and other tissues with high lipid content, such as the brain and nervous system (Soleas et al., 2001). Consequently, the neuroprotective action of resveratrol has been investigated by several groups in different models of neurological illness (Anekonda, 2006; Gélinas and Martinoli, 2002; Okawara et al., 2007). In fact, various points of evidence provide interesting insights into the effect of resveratrol in neurological-related diseases such as Alzheimer and Parkinson (Brown et al., 2009; Hall et al., 2010; Lee et al., 2007). Accordingly, one interesting result observed in this work was the protective effect exhibited by resveratrol in the fluphenazine induced OD, a model of extrapyramidal syndrome which has some features similar to TD which is highly prevalent in humans taking neuroleptic treatment. Here we demonstrate, for the first time, that the resveratrol treatment reduced VCM prevalence in the animals acutely treated with fluphenazine. On the order hand, resveratrol did not blunt the intensity of the VCM in those animals that developed +VCM. Regarding mechanisms, our group has previously demonstrated a reduction in dopamine uptake in animals presenting high intensity of VCMs after chronic treatment with haloperidol or fluphenazine (Fachinetto et al., 2007a, 2007b). In line with this, other studies have indicated that resveratrol can increase dopamine levels and dopamine transporter in striatum (Di Liberto et al., 2012). Recently, our group has demonstrated that resveratrol prevented the development of VCM in an animal model of parkinsonism induced by reserpine in mice (Busanello et al., 2011). Taken together, these data support the idea that the neuroprotective effect exhibited by resveratrol may be associated with modulation of dopaminergic system. It is important to emphasize that resveratrol prevented VCM in different animal models, which account to promissory use of resveratrol to treat parkinsonism and TD. This effect of resveratrol in attenuating the VCMS in both orofacial dyskinesia models suggests that it is probably acting at common points to both models, different from *Valeriana officinallis* that was only effective in reversing VCM induced by reserpine in rats (Fachinetto et al., 2007b; Pereira et al., 2011). Consistent with these findings, an elegant study demonstrated that resveratrol treatment restore the dopamine transporter protein expression after $MPTP^+$ induced toxicity in PC12 cells (Gélinas and Martinoli, 2002). Moreover, the neuroprotection exhibited by resveratrol in our experimental protocol may be related with its antioxidant property, since there are points of evidence in the literature showing that dopamine causes apoptosis in SH-SY5Y cells through the induction of oxidative stress pathways and that this deleterious effect is attenuated by resveratrol pre-treatment (Lee et al., 2007). Although we have previously not detected oxidative stress after chronic and acute fluphenazine treatment, it could be occurring in specific areas and can spread to other brain areas depending on subtle factors that were not controlled in these studies (Fachinetto et al., 2007a). Thus, resveratrol possibly may be acting in different molecular pathways to avoid VCMs in rats.

It has been previously published that cis- and trans-resveratrol treatment reduces noradrenaline and 5-hydroxytryptamine uptake, monoamine oxidase activity (Yáñez et al. 2006); which, consequently, could increase the locomotor and exploratory activity caused by activation of these systems. Thus, we have also evaluated in this work the locomotor and exploratory performance of animals in the open field test. It is known that de blockage of D_2 and to a less extent D_1 receptors cause a decrease in locomotor and exploratory behavior (Redrobe et al., 1998). As expected, we found a reduction in locomotor and exploratory activity in rats under acute treatment with fluphenazine, a neuroleptic that causes mainly a blockage of D_2

receptors. However, these behavior alterations were not ameliorated by resveratrol. Accordingly, a recent study of our group demonstrated that resveratrol did not blunt the hypolocomotion caused by reserpine in mice (Busanello et al., 2011).

Taken together, our work is the first to demonstrate a beneficial role of resveratrol use in an acute animal model of OD fluphenazine-induced. However, additional studies are needed to elucidate the precise mechanisms involved in the neuroprotective effects exhibited by resveratrol in this experimental model.

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3.2 MANUSCRITO 1

RESVERATROL INHIBITS THE ACTIVITY OF MONOAMINE OXIDASE: A COMPARATIVE *IN VITRO* AND *IN SILICO* STUDY

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Abstract

Resveratrol, a polyphenol compound present in red grapes and red wine, is recognized for its neuroprotective effects. In this study, we evaluated the inhibitory potential of *trans*-resveratrol on *in vitro* MAO-A and MAO-B activities from rat brains. In addition, we performed molecular docking studies with isomers *cis* and *trans*-resveratrol to better understand the mechanisms involved. We found that resveratrol inhibited both MAO isoforms (iC_{50} values were 1.599 µg/mL and 44.45 µg/mL for MAO-A and MAO-B, respectively) with approximately 28-fold higher potency toward MAO-A. Regarding the nature of MAO-A and MAO-B inhibition, resveratrol showed to be a noncompetitive agent for both enzymes. Furthermore, a profile partially reversible for MAO-A and completely reversible for MAO-B was obtained. The *in silico* study on human enzyme showed that *cis*-resveratrol and *trans*-resveratrol interacted in the active site of MAO-A and MAO-B respectively, preventing the entrance of substrate. The formation of H-bonds between enzyme and hydroxyl groups from resveratrol can be responsible for the affinity with them. These results suggest that resveratrol is a MAO inhibitor and, at least in part, the neuroprotective activity of it could be attributed to this effect.

Keywords: MAO inhibitor, *trans*-resveratrol, docking, rat brain

Introduction

Monoamine oxidase (MAO) catalyses the oxidative deamination of biogenic amines, such as serotonin, norepinephrine and dopamine, and it seems to play important role in several psychiatric and neurological conditions (Youdim et al., 2006). MAO is a flavor-protein with flavin adenine dinucleotide (FAD) as the cofactor and is located at the outer of mitochondrial membrane of monoaminergic neurons, glia, and other cells. Considering pharmacological, biochemical and genetic studies, two isoforms of MAO were characterized: MAO-A and MAO-B (Bach et al., 1988; Johnston, 1968), which exhibit about 70% of homology (Shih et al., 1999). MAO-A preferentially deaminates serotonin and is inhibited by low concentrations of clorgyline, whereas MAO-B deaminates phenylethylamine and benzylamine and is inhibited by low concentrations of selegiline. Epinephrine, norepinephrine, dopamine, tryptamine and tyramine are oxidized by both isoforms in most species (Youdim et al., 2006).

Monoamine oxidase inhibitors (MAOIs) were the first antidepressant drugs described and are still used nowadays with relative success, especially in patients resistant to other treatments (Berton and Nestler, 2006; López-Muñoz et al., 2007; Thase et al., 1995; Wong and Licinio, 2004). The antidepressant properties result from selective MAO-A inhibition in the central nervous system (López- Muñoz et al., 2007; Robinson, 2002). Furthermore, MAO-B inhibitors have been used with successes to treat patients with Parkinson's disease (Youdim and Bakhle, 2006) since the basal ganglia from human brain had predominantly MAO-B (Collins et al., 1970; Youdim et al., 1972). The reaction catalyzed by MAO produces hydrogen peroxide, a source of hydroxyl radicals, and MAO inhibitors might, therefore, be useful in managing the outcome of stroke and other tissue damage associated with oxidative stress (Bianchi et al., 2005; Youdim et al., 2006). However, the MAOIs that are being used present a variety of undesirable effects, including the hypertensive crises that may be induced when given in combination with foods containing tyramine. These particularities drive the search for new MAOIs that exhibit a reversible and selective profile.

In this context, some natural products have been investigated by their antidepressant and/or antiparkinsonian properties (Guo et al., 2016; Xu et al., 2016; De Oliveira et al., 2015). Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a natural phenolic compound present in grapes, cranberries, and peanuts that exists as *cis*- and *trans*-isomers, due the double bond in its chemical structure (Aggarwal et al., 2004; Frémont 2000; Orallo 2006; Pervaiz 2003). Most of pharmacological studies have considered the *trans* isomer, which has been suggested to be one of the principal wine components and responsible for the cardioprotective effects from

resveratrol (Aggarwal et al., 2004; Bradamante et al., 2004; Delmas et al., 2005; Hao and He 2004; Olas et al., 2005; Wu et al., 2001). Several beneficial properties have been attributed to resveratrol including antidepressant (Liu et al. 2016; Xu et al., 2010) and antiparkinsonian (Xu et al., 2016). These pharmacological effects have been associated with resveratrol ability in modulating biogenic amines levels (Blanchet et al., 2008). In this sense, some studies have demonstrated that resveratrol is able to inhibit MAO isoforms (Yáñez et al., 2006). However, the kinetic and reversibility of reaction have not been investigated yet.

Considering the inhibitory effect of resveratrol on MAO activity, this study was aimed to determine kinetic parameters and the reversibility of the reaction. Furthermore, we also use the Vina program to understand how resveratrol isomers interact in MAO enzymes and cause inhibition, using human isoforms.

Materials and Methods

Animals

Adult male Wistar rats, weighing 200 to 220 g, from breeding colony of UFSM were kept in cages (4-5 animals per cage) with continuous access to food and water. The room housing of the cages was temperature-controlled (22 ± 2°C), on a 12-h light/dark cycle with the lights going on at 7:00 am. The experimental procedure was previously approved by the Ethical Commission of Animal Use from Federal University of Santa Maria under number 091/2013.

Drugs

Resveratrol (3,4,5-trihydroxy-trans-stilbene from Chengdu Hawk Bio Enginnerin, China) was obtained from commercial pharmacy. All other reagents were obtained from Sigma-Aldrich.

Tissue Preparation

Rats were euthanized by decapitation. The brain was homogenized in assay buffer (16.8 mM Na_2HPO_4, 10.6 mM KH_2PO_4, and 3.6 mM KCl, pH 7.4).

Kinetic parameters of MAO inhibition determination in vitro

The inhibitory potential of resveratrol on MAO-A and MAO-B activities was dertermined in rat brain preparations by a fluorimetric method using kynuramine as a substrate,

as previously described (Villarinho et al., 2012; De Oliveira et al., 2015; De Freitas et al., 2016). Activities of A and B isoforms were pharmacologically isolated by incorporating 250 nM pargyline (selective MAO-B inhibitor) or 250 nM clorgyline (selective MAO-A inhibitor) into the reaction mix. The reaction mixture (containing brain homogenates, resveratrol and inhibitors) was pre-incubated at 37°C for 10 min, and the reaction was started by addition of 50 µL of kynuramine (90 µM for MAO-A and 60 µM for MAO-B). Resveratrol was tested at concentrations ranging from 0.03 to 300 µg/mL. IC_{50} and K_i values for both MAO isoforms were determined. Assays were performed in duplicate in a final volume of 500 µL containing 0.25 mg of protein and incubated at 37ºC for 30 min.

For kinetic experiments, various concentrations of kynuramine (1–100 µM) were used, and MAO-A and MAO-B activities were determined in the absence or presence of different concentrations of resveratrol (0.1-100 µg/mL) in order to calculate K_m and V_{max} values. The reversibility of MAO-A and MAO-B inhibition was evaluated using dialysis method as described by Harfenist et al. (1996) with minor modifications. Mixtures of buffer, brain homogenates and pargyline or clorgyline with and without resveratrol (10, 30 or 100 µg/mL, for MAO-A and MAO-B respectively) were dialyzed at 25°C for 24 h. In the control mixture, 0.1% ethanol was added. For each 1 mL of mixture dialyzed, 40 mL of outer buffer (16.8 mM Na_2HPO_4, 10.6 mM KH_2PO_4, 3.6 mM KCl, 1 mM dithiothreitol) was used. The outer buffer was replaced with fresh buffer at 1, 2, 4 and 6 h after the start of dialysis. Nondialyzed portions of each mixture were maintained at 25°C over the same time period.

Twenty-four hours after the start of dialysis, dialyzed and nondialyzed mixtures were simultaneously assayed for MAO-A or MAO-B activity in order to evaluate the reversibility of the inhibition produced by resveratrol.

Molecular Docking

In silico molecular docking studies have been widely used as important tools to understand the interaction between ligands and enzymes. The AutoDockVina 1.1.1 is a free-academic docking program that operates by pairing an empirically-weighted scoring function containing terms for values such as hydrogen bonding, van der Waals interactions, rotatable bond penalties, and a sophisticated gradient-based local search as a global optimization algorithm (Trott et al. 2010). The binding free energy (ΔG) generated by AutoDockVina is the thermodynamic magnitude that provides whether the process is spontaneous ($\Delta G < 0$) or not ($\Delta G > 0$).

In order to ensure the efficiency of Vina 1.1.1 program in proposing a reliable molecular model in our study, a previous redocking (by a blind docking) of the crystal ligand from MAO-A (PDB: 2Z5Y) and MAO-B (PDB: 3PO7) was performed, using an exhaustiveness of 200, and grid box coordinates, x=-34.79, y=-27.701, z=-16.372 (dimensions of the grid box: 74Å x 62Å x 64Å) for MAO-A, and for MAO-B was used: x=53.012, y=143.625, z=29.039 (dimensions of the grid box: 58Å x 76Å x 72Å).

Previously, in both enzyme structures, the molecules of water, ions, ligands and other molecules were removed (excepting the cofactor FAD). *Cis*- and *trans*-resveratrol molecules were created using the software Avogadro 1.1.1 (Hanwell et al., 2012), following the semi empirical PM6 (Stewart, 2007) geometry optimization using the program MOPAC2012 (Stewart, 2012). The enzymes, original ligands and resveratrol isomers were prepared for the docking, using the AutoDock Tools 4.2 (Morris et. al., 2009), where the compounds were considered flexible, and the MAOs enzyme rigid (with Gasteiger charges). The polar hydrogens were added in both cases. Resveratrol isomers docking was performed using the same configuration of the redocking. The structure with the most favorable binding free energy was selected and analyzed using the Accelrys Discovery Studio 3.5.

Statistical analyses

Results from *in vitro* study were expressed as means±S.E.M., except for K_i values, which were reported as means followed by 95% confidence intervals. IC_{50} values were calculated by nonlinear regression using sigmoidal dose–response with a variable slope equation, whereas K_m (µg/mL) and V_{max} (nmol/min/mg protein) values were calculated by nonlinear regression using the Michaelis–Menten equation. Apparent K_i values were calculated using the following equation: $K_i = IC_{50}/[1+([S]/Km)]$ (Cheng and Prusoff, 1973). Differences between groups were evaluated for significance using one-way analysis of variance (ANOVA) followed by Tukey's test or unpaired t-test. Significance was considered when $p<0.05$.

Results

Effects of resveratrol on MAO-A and MAO-B activity

Resveratrol inhibited both MAO isoforms in a concentration-dependent manner with iC_{50} values of 1.599 (1.2–2.1) µg/mL and 44.45 (31.5–62.6) µg/mL for MAO-A and MAO-B, respectively (Figure 1A and 1B). Furthermore, the Ki values found were 0.336 (0.276-0.314) µg/mL for MAO-A and 7.92 (7.44-8.66) µg/mL for MAO-B.

Effects of resveratrol on kinetic of MAO-A and MAO-B

Subsequently, kinetic experiments for isoforms of MAO were carried out using different concentrations of substrate and resveratrol. Resveratrol did not alter the K_m value for MAO-A (Figure 2A and Table 1) and MAO-B (Figure 2B and Table 1) when compared to K_m values obtained in the absence of resveratrol. However, resveratrol caused a decrease in the V_{max} value for MAO-A at concentrations of 1 and 3 µg/mL (Figure 2A and Table 1). For MAO-B this effect of resveratrol on V_{max} was observed at concentrations of 10, 30 and 100 µg/mL (Figure 2B and Table 1).

Reversibility of MAO activity in the presence of resveratrol

We evaluated the reversibility of the MAO-A and MAO-B inhibition induced by resveratrol using the dialysis method. Figure 3A shows that the inhibition of MAO-A activity produced by resveratrol was partially reversed after 24 h of dialysis when compared with the respective nondialyzed control. Differently, the inhibition of MAO-B activity produced by resveratrol was completely reversed after 24 h of dialysis compared to the respective nondialyzed control (Figure 3B).

Molecular docking results

Redocking values obtained for MAO-A and MAO-B were of 1.74 Å and 1.03Å, respectively (Figure 4). MAO-A and B docking results showed that resveratrol isomers interacted in distinct regions from proteins. Figure 5A illustrates that *cis*-resveratrol (blue) interacts with the active site of MAO-A in the same region of the crystal ligand (pink), close to the cofactor FAD (orange), interacting with Asn181, Tyr444 and FAD by hydrogen bonds (H-bond) (Figure 6A). *Trans*-resveratrol (yellow) bound to another region making a cation-π interaction with Arg129, a π-π stacking with His488, and an H-bond with Asn125 (Figure 6B). For MAO-B protein (Figure 5B), *trans*-resveratrol interacted in the site of the crystal ligand, making a π-π stacking with Tyr398 and Tyr435, and an H-bond with nitrogen from FAD (Figure 6D). The values of ΔG (Table 2) demonstrated that in MAO-A both resveratrol isomers interacted with practically the same affinity, but in MAO-B *trans*-resveratrol presented a higher affinity when compared with the *cis*-resveratrol and crystal ligand Zonisamide.

Discussion

Literature data have demonstrated that resveratrol possess many pharmacological properties including antidepressant and antiparkinsonian, activities that might encompass MAO

inhibition. However, the mechanisms involving MAO enzyme have not well understood. In the present study, we evidenced that resveratrol inhibited *in vitro* MAO-A and MAO-B, in the homogenized rat brain, with a higher potency towards MAO-A. In both cases resveratrol caused a reduction in V*max* without alter the -K*m* of reaction. Furthermore, the inhibition of MAO-A was partially reversible while MAO-B completely reversible. In *in silico* study, using human MAO-A and *cis*-resveratrol and MAO-B and *trans*-resveratrol, both isomers interacted in the active site of the enzymes, preventing the entrance of substrate.

Resveratrol has been largely studied due its numerous pharmacological properties (Park and Pezzuto 2015), especially the neuroprotective effect attributed to antioxidant activity (López-Miranda et al., 2012). Considering the actions of resveratrol in the central nervous system, some findings have demonstrated the antidepressant (Liu et al., 2016 Xu et al., 2010) and antiparkinsonian (Xu et al., 2016) effects of compound in animal models. In accordance, previous studies from our group have demonstrated that resveratrol is able to reduce vacuous chewing movements induced by reserpine (Busanello et al., 2011) or fluphenazine (Busanello et al., 2012). We suppose, that these effects, at least in part, are related with the role of compound towards monoamines and oxidative stress. In this scenario, MAO enzyme highlight as an important target in neurodegenerative disorders, mainly in Parkinson's disease (Youdim and Bakhle, 2006). MAO has two isoforms, MAO-A and MAO-B, distributed along of tissues. The inhibition of MAO-B is related with Parkinson's disease improvement (Youdim and Bakhle, 2006) while MAO-A inhibition with antidepressant activity (Liu et al., 2016; Xu et al., 2010) and protection against cell apoptosis (Ou et al., 2006). MAO catalyzes the oxidative deamination of biogenic amines (Youdim and Bakhle, 2006) and its inhibition decreases the formation of acidic metabolites and hydrogen peroxide which is related to neuroprotective effects by antioxidant mechanisms (Youdim and Bakhle, 2006). In this sense, there is evidence that *cis*- and *trans*-resveratrol reduced the noradrenaline and 5-hydroxytryptamine uptake and inhibited the activity of both isoforms of MAO (Yáñez et al., 2006). However, Zohu et al., (2001) demonstrated that resveratrol inhibited only the activity of MAO-A of rat brain mitochondria. Considering these findings, the first aim of this study was to confirm the ability of resveratrol in inhibiting MAO activity and determine kinetic parameters and the reversibility of reaction. Resveratrol inhibited both isoforms of MAO with a potency approximately 28 times higher towards MAO-A than MAO-B. These data are in accordance with a previous study which demonstrated that resveratrol inhibits MAO-A in a minor concentration than that necessary to inhibit MAO-B (Yáñez et al., 2006). We also evaluated the kinetic profile of MAO inhibition by resveratrol. Resveratrol did not alter the K_m value of both MAO isoforms.

However, it caused a decrease in the V_{max} value for MAO-A and MAO-B compared to V_{max} values obtained in the absence of resveratrol, results that characterize a noncompetitive inhibition. Indeed, we analyzed whether the binding of resveratrol in MAO enzyme was reversible or irreversible. It was observed that after 24 hours of dialysis the activity of MAO-A was partially recovered being dependent on the concentration of resveratrol used. Regarding MAO-B, the activity returned to values upper than 80% which is considered an inhibition completely reversible (Harfenist et al., 1996). To our knowledge this was the first study performed toward kinetic and reversibility parameters of resveratrol on MAO activity. Considering the present data, it is possible suppose that part of the neuroprotective effects elicited by resveratrol are due to its ability of inhibiting MAO activity and consequently diminish oxidative events.

The second aim of this study was to understand how resveratrol isomers could be interacting in the enzyme and causing inhibition. For this, we apply Vina program using a model with the crystallized structure of human MAO. Molecular docking studies were performed with both isoforms of resveratrol to rationalize their possible interactions with the MAO. The redocking results indicated that the procedure used here was a good model for predict the interaction between receptor and target, because the RMSD values obtained for MAO-A and MAO-B was of 1.74 Å and 1.03Å, respectively, and according with Bursulaya (2003) RMSD value less than 2 Å is a good condition for the correct bound structure prediction. According MAO-A and B docking results, in both cases resveratrol isomers interacted in distinct regions of proteins. *Cis*-resveratrol (blue) interacted in the active site of MAO-A, in the same region of the crystal ligand (pink), close to the cofactor FAD (orange), interacting specifically with Asn181,Tyr444 and FAD by hydrogen bonds (H-bond). The presence of Tyr444 is of great importance in the MAO-A active site, because it together with Tyr407 and FAD form the aromatic cage, that are involved in the stabilization of aromatic compounds, by π-π stacking interaction (Karuppasamy et al., 2010; Beula et al., 2014), although not present in this case. *Trans*-resveratrol (yellow) bound to another region, close to the membrane-binding motif in C-terminus from MAO-A (Wang et. al., 2013), making a cation-π interaction with Arg129, a π-π stacking with His488, and an H-bond with the Asn125. For MAO-B protein, impressively the opposite occurred; *trans*-resveratrol interacted in the site of the crystal ligand, making a π-π stacking with Tyr398 and Tyr435, and an H-bond with the nitrogen from FAD. The presence of π-π stacking in the Tyr398 and Tyr435 demonstrated its importance in the stabilization of ligand in the active site by hydrophobic interactions. Similar results were demonstrated by Mustafa Toprakçí and Kemal Yelekçi (2005), Simona Distinto et al (2012) and Kare *et al*

(2013). On the other hand, *cis*-resveratrol, bound in an equivalent region of MAO-A (when compared visually), close to the C-terminus from protein, interacting with the residues Asp123 and Asn116 by H-bonds. The ΔG values obtained demonstrated that in MAO-A both resveratrol isomers interact with similar affinity, but in MAO-B the *trans*-resveratrol presents higher affinity when compared with the *cis*-resveratrol and crystal ligand Zonisamide. In 2013, Chi Chiu Wang *et al* demonstrated that both MAO-A and B present 73% of residues conserved, this little difference can be involved with the distinct forms of resveratrol isomers in interacting with the enzymes.

The variation found towards the binding local in the *in vitro* and *in silico* studies could be due to differences between rat MAO-A and human MAO-B. It is known that one of the main differences between rat MAO-A and human MAO-B is the shape and configuration of their respective substrate-binding sites. A significant distinction in the structures of human and rat MAO-A is the conformation of the cavity-shaping loop formed by residues 210–216 (De Colibus et al., 2005). In rat MAO-A this loop is unlike that found in the human MAO-A enzyme, although it is the same as the homologous loop in human MAO-B. This difference is considered responsible for smaller volume of the active site cavity from rat MAO-A (Youdim et al., 2006; Edmondson et al., 2009).

Conclusion

Taken together, the results show that *trans*-resveratrol presents inhibitory effects on MAO-A and MAO-B activity *in vitro*, which were observed also *in silico* study using human isoforms of MAO enzyme. In addition, *trans*-resveratrol exhibits a noncompetitive profile of inhibition that is totally reversible for MAO-B and partially reversible for MAO-A. Comparing *in silico* results, *cis*-resveratrol in MAO-A enzyme and *trans*-resveratrol, in MAO-B, both interact in the active site of the enzyme, preventing the entrance of substrate. We highligth here the importance of hydroxyl groups from resveratrol in the H-bonds with the enzymes, which can be responsible for the affinity between them. Collectivelly, our data point resveratrol as a promising agent for adjuvant therapies on Parkinson's disease and depression due to its ability in inhibiting MAO activity.

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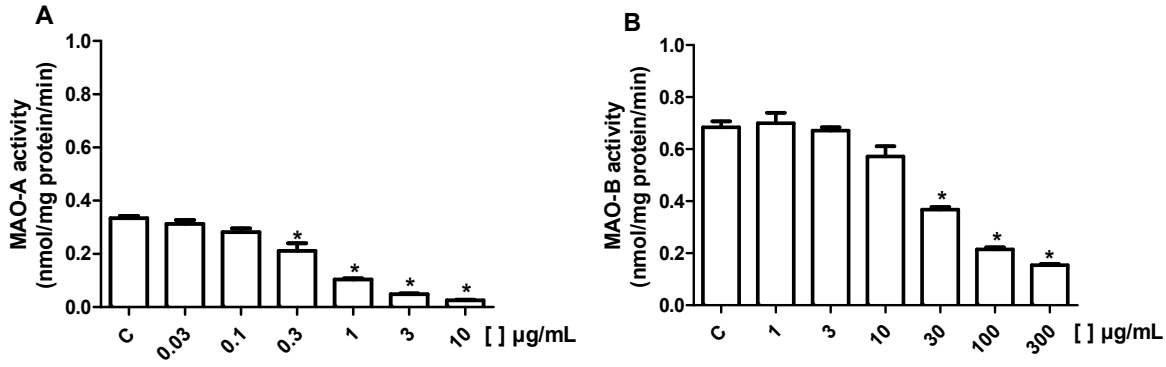

Figure 1: Inhibitory potential of resveratrol on in *vitro* (A) MAO-A and (B) MAO-B activities in the mitochondrial fraction of rat brain. Values are means±S.E.M. of three experiments performed in duplicate. One-way ANOVA followed by Tukey's test. *Significant differences from control.

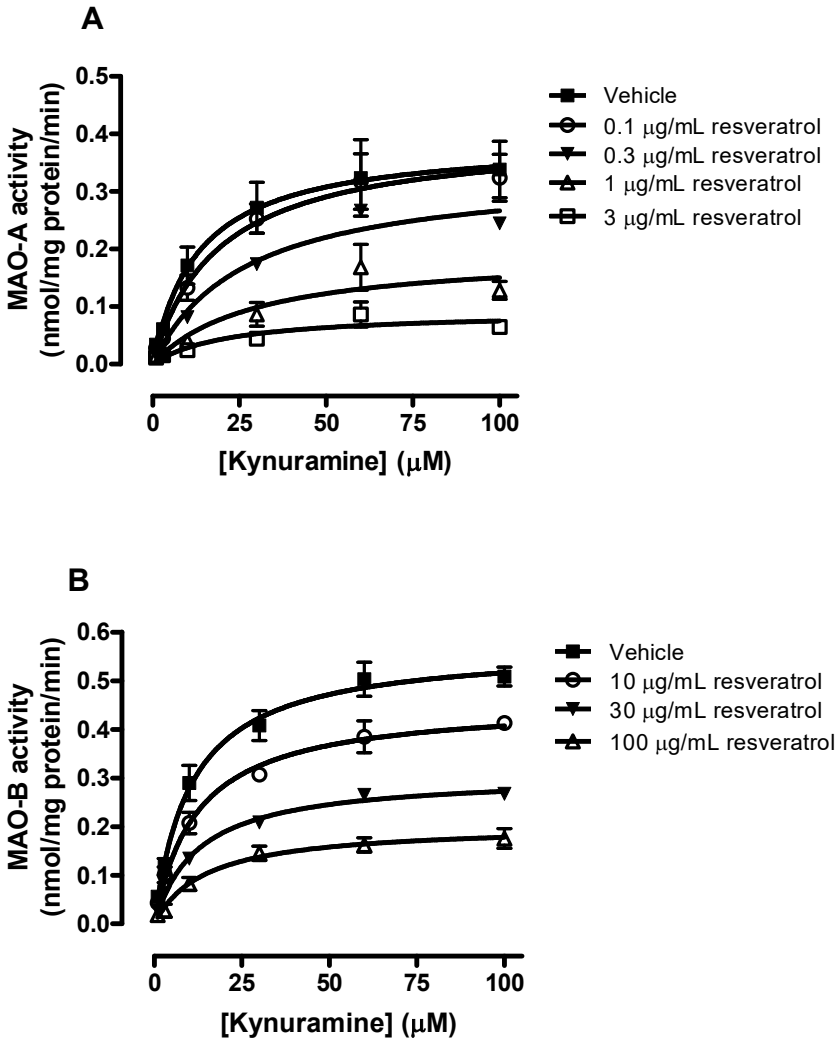

Figure 2: Substrate concentration curves for *in vitro* (A) MAO-A and (B) MAO-B activity in the absence or presence of resveratrol in the mitochondrial fraction of rat brain. Values are means±S.E.M. of three experiments performed in duplicate.

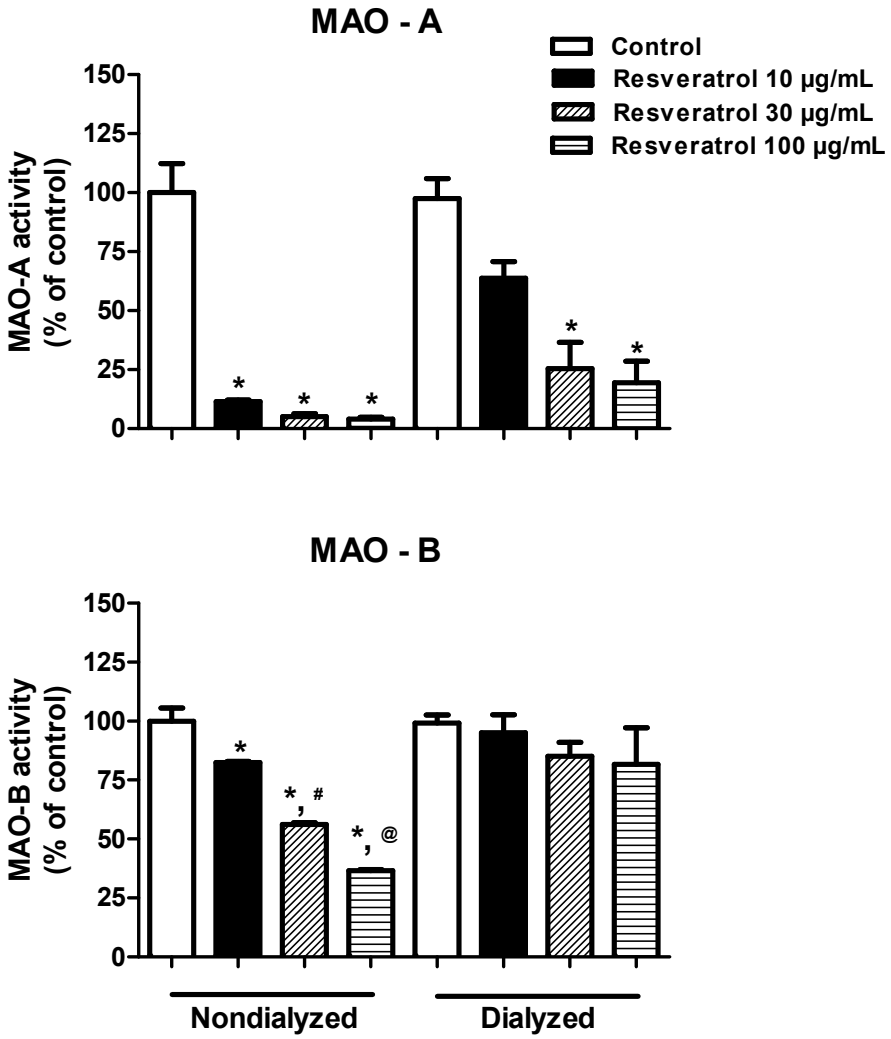

Figure 3: Reversibility of MAO-A (A) and MAO-B (B) inhibition produced by resveratrol after 24 h of dialysis. Values are means±SEM of three to four experiments performed in duplicate. Data were analyzed by t-test. *$p<0.05$ compared with its respective control; $^{\#}p<0.05$ compared with resveratrol 10 µg/mL; $^{@}p<0.05$ compared with resveratrol 10 µg/mL and 30 µg/mL.

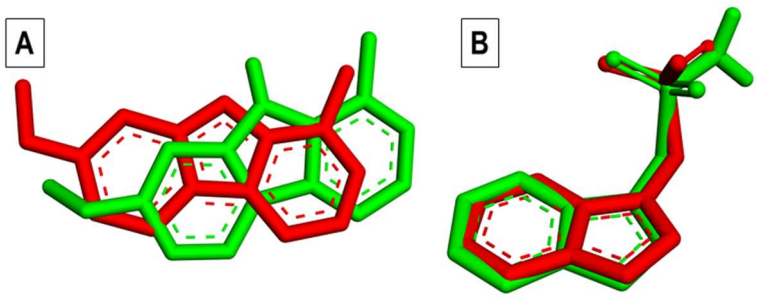

Figure 4: Redocking results for MAO-A (A - Harmine) and MAO-B (B - Zonisamide) (1.74 Å and 1.03Å, respectively). The molecules were overlaid, and in red is represented the original position of ligand from PDB crystal, and in green showed the docking results. The non-polar hydrogens were omitted.

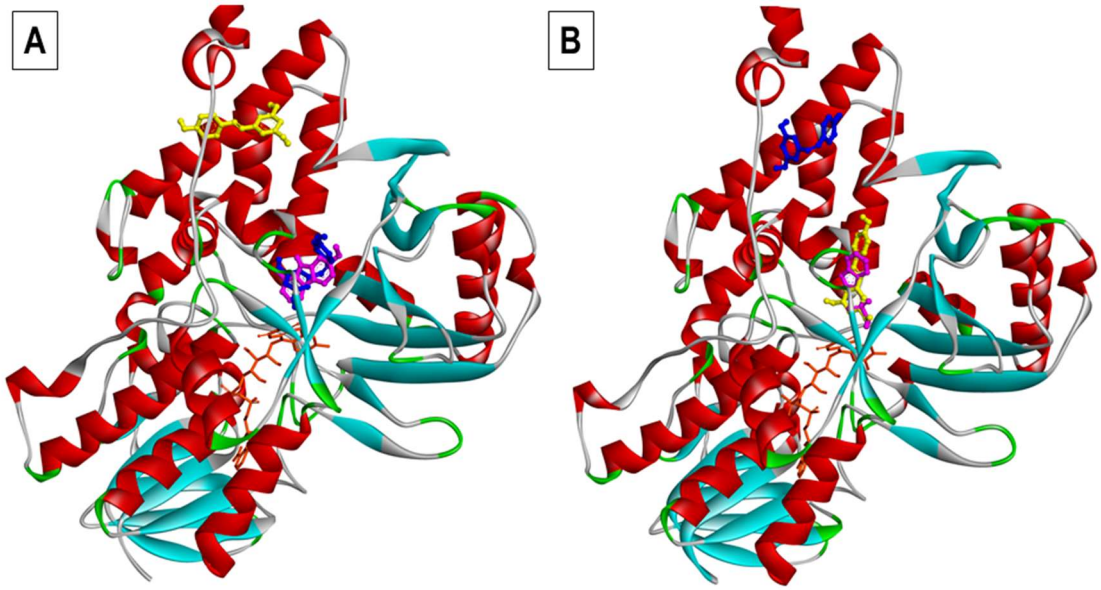

Figure 5: Comparison between the positions of the ligands and the resveratrol isomers in MAO-A (A) and MAO-B (B) structures. The original crystal ligand is showed in pink, cis-resveratrol in blue, trans-resveratrol in yellow and the cofactor FAD in orange.

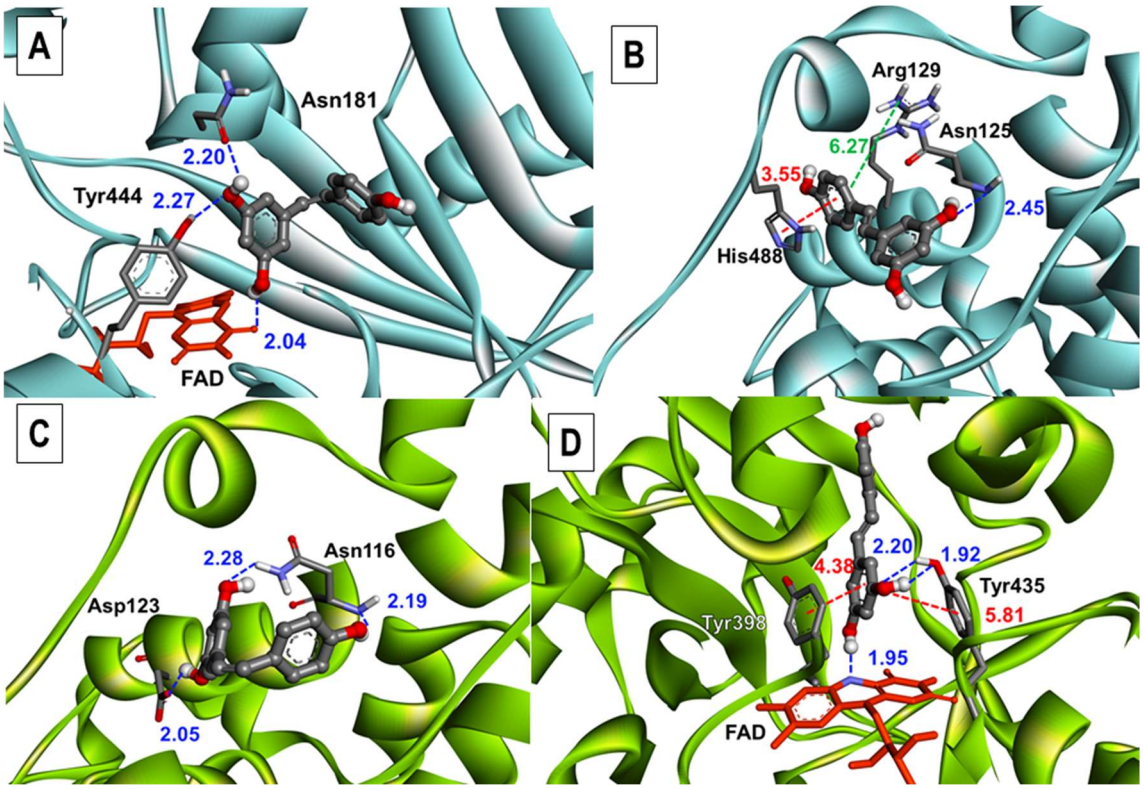

Figure 6: Docking results for MAO-A (cyan) and MAO-B (green). The cis-resveratrol (A and C) and trans-resveratrol (B and D) is showed in ball and stick. The residues and the cofactor FAD in stick. In red dot lines is showed the π-π stacking, in green dot lines cation-π stacking, and in blue dot lines H-bonds. The distances of the interactions is demonstrated in Å, in its respectively colors.

	Resveratrol (µg/mL)	Vmax (nmol/min/mg protein)	Km (µM)
MAO-A	0	0.3886±0.0437	13.12±5.35
	0.1	0.3999±0.0405	18.79±6.13
	0.3	0.3369±0.0466	26.41±10.37
	1	0.1945±0.0338*	29.28±13.90
	3	0.0918±0.0143*	22.22±10.47
MAO-B	0	0.5702±0.0260	10.44±1.85
	10	0.4559±0.0203*	12.07±2.00
	30	0.3064±0.0112*	12.47±1.68
	100	0.2037±0.0101*	14.52±2.54

Table 1: Effects of different concentrations of resveratrol on K*m* and V*max* values of MAO-A and MAO-B activity. Data were analyzed by t-test. *Represents significant difference from control without resveratrol.

Enzyme	Molecule	ΔG (kcal.mol^{-1})
MAO-A	Harmine*	-8.1
	cis-resveratrol	-7.4
	trans-resveratrol	-7.5
MAO-B	Zonisamide*	-6.8
	cis-resveratrol	-7.9
	trans-resveratrol	-8.7

Table 2: Binding free energy (ΔG) for the resveratrol molecules and the ligands from MAO-A and B, obtained from Vina 1.1.1. *Original ligands from MAO-A (2Z5Y)and MAO-B (3PO7), respectively.

3.3. MANUSCRITO 2

RESVERATROL PROTECTS AGAINST VACUOUS CHEWING MOVEMENTS INDUCED BY CHRONIC TREATMENT WITH FLUPHENAZINE

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Abstract

Typical antipsychotics, which are commonly used to treat schizophrenia, cause motor disorders as tardive dyskinesia (TD) in humans and orofacial dyskinesia (OD) in rodents. The disease mechanisms as well as treatment effectiveness are still understood. In this study, we investigated the effect of resveratrol, a polyphenol with neuroprotective properties, on behavioral changes induced by chronic treatment with fluphenazine in rats and the possible relation between MAO activity and vacuous chewing movements (VCMs). Rats were treated during 18 weeks with fluphenazine enantate (25 mg/Kg, i.m., each 21 days) and/or resveratrol (20 mg/Kg, offered daily in drinking water). After, body weight gain, behavioral parameters (VCMs and open field tests – locomotor and rearing activity) and MAO activity were evaluated. The treatment with fluphenazine reduced the body weight gain, number of crossings and rearings and the co-treatment with resveratrol did not avoid these alterations. Fluphenazine increased the VCMs and the co-treatment with resveratrol reduced the intensity of VCMs. Furthermore, a negative correlation was found between the number of VCMs and MAO-B activity in striatum of rats. Our data suggest that the reduction in MAO-B activity is related to VCMs intensity and that chronic treatment with resveratrol could be promising to decrease OD.

Keywords: tardive dyskinesia, orofacial dyskinesia, resveratrol, MAO enzyme.

1. Introduduction

Schizophrenia is one of the most debilitating psychiatric disease which affects approximately 1% of the population (Sawa and Snyder, 2002; Kahn et al., 2015). The complexity of disease makes the treatment quite difficult, being typical and atypical antipsychotics the more effective drugs used nowadays (Snyder and Vanover, 2014). The pharmacological mechanism of typical antipsychotics, such as fluphenazine, involves the blockage of dopamine D_2 receptors in limbic areas (Creese et al., 1976; Seeman et al., 1976; Carlson, 1988). However, they also act in the nigrostriatal pathway causing debilitating motor effects like tardive dyskinesia (TD) (Casey, 1985).

TD is characterized by hyperkinetic, repetitive and involuntary movements of orofacial region, affecting also the neck, limbs (especially the upper) and trunk (Crane, 1973; Glazer et al., 1990; Andreassen and Jørgensen, 2000). It may occur during or after discontinuation of chronic treatment with antipsychotics and might be irreversible even after antipsychotic withdrawal (Andreassen and Jørgensen, 2000). Some authors estimate that the prevalence of TD in patients receiving treatment with typical antipsychotics is around 20-25% (Lee et al., 2014), rate that increases with age and reach approximately 50% of patients over 50 years old (Kane and Smith, 1982; Gardos and Cole, 1983; Yassa and Jeste, 1992). Athough there are many researchers investigating the mechanisms involved in TD and/or possible treatments, few progress has been made in this aspect.

Dopaminergic alterations with consequent production of reactive oxygen species have been proposed as a possible mechanism involved in development of TD in humans and orofacial dyskinesia (OD) represented by vacuous chewing movements (VCMs) in animals (Rana et al., 2013). In this scenario, some authors have highlighted the role of enzyme monoamine oxidase (MAO) (Lohr, 1991; Lohr et al., 2003). MAO participates in metabolism of monoamines, including dopamine (DA), norepinephrine and serotonin (Youdim et al., 2006). Literature reports show that the administration of antipsychotics and the consequent blockage of dopamine receptors increases dopamine synthesis and its metabolism by MAO (Lohr, 1991; Andreasen and Jørgensen, 2000). Increased DA metabolism by MAO culminates with hydrogen peroxide (H_2O_2) overproduction, which might react with transition metals via Fenton reaction, generating reactive species such as hydroxyl ($OH^{\cdot}$) and anion superoxide (O^{-}_{2}) radicals (Youdim and Bakhle, 2006). In addition, DA can itself undergo autoxidation to form quinones of dopamine, potent oxidant species (Lohr et al., 2003; Lohr, 1991). A recent study demonstrated that the increase in VCMs induced by reserpine in mice is associated with a reduction in MAO activity, indicating the important role of enzyme towards OD (Freitas et al., 2015). On the other hand,

some data from literature point a relative effectiveness of MAO inhibitors in experimental models of OD (Sachdev et al., 1999).

Resveratrol is a phytoalexin found in grapes, cranberries, and peanuts (Pervaiz and Holme 2009) that exhibits antioxidant properties. In addition, resveratrol plays several pharmacological effects, including neuroprotection (Gehm et al., 1997; Gelinas and Martinoli, 2002; Quincozes-Santos and Gottfried, 2011; Pallàs et al., 2013). Of particular importance to our study, there is evidence that resveratrol may modulate some proteins of dopaminergic system including MAO (Yáñez et al., 2006; Di Liberto et al., 2012). In this sense, our group recently showed that acute exposute to low doses decreases both VCMs induced by reserpine in mice (Busanello et al., 2011) and VCMs induced by fluphenazine in rats (Busanello et al., 2012). However, studies have demonstrated that some substances that have promissory effects on acute models of OD does not have the same efficacy on chronic models (Fachinetto et al., 2007a, b; Burger et al., 2006; Pereira et al., 2011). Chronic models of OD present more similarities with TD than acute models which are characterized as extrapyramidal syndrome (Andreasen and Jørgensen, 2000). In this model, there are neither data about the role of resveratrol against VCMs induced by chronic treatment with fluphenazine nor the participation of MAO. Considering such aspects, the aim of this study was to investigate the effects of chronic treatment with resveratrol on behavioral changes induced by chronic treatment with fluphenazine in rats and the involvement of MAO activity on VCMs.

2. Materials and Methods

2.1 Animals

Adult male *wistar* rats (60 days old), weighing 200-220g, were purchased from breeding colony of UFSM and kept in cages (five animals) with food and *water ad libitum.* The room housing was temperature-controlled (22 ± 2°C) and on a 12-h light/dark cycle with the lights on at 7:00 am. The experimental procedure was previously approved by the Ethical Commission of Animal Use from Federal University of Santa Maria under the number of 051/2011.

2.2 Drugs

Fluphenazine enantate (Flufenan® from Cristália) and resveratrol (3,4,5-trihydroxy-trans-stilbene) were commercially acquired from Chengdu Hawk Bio Enginnerin, China. All other reagents were obtained from Sigma-Aldrich or other companies that guarantee purity and quality of their products.

2.3 Experimental protocol

The rats were divided into four groups: control (n=5), resveratrol (n=5), fluphenazine (n=9) and fluphenazine plus resveratrol (n=8). The treatment with resveratrol and/or fluphenazine was carried out during 18 weeks (Fachinetto et al. 2007a). Fluphenazine enantate (25 mg/Kg) or its vehicle (soy oil, 1 mL/Kg) were administered every 21 days by intramuscularly (i.m.) route (Fachinetto et al., 2007a; Busanello et al., 2012). Concomitantly, resveratrol (20 mg/kg) or its vehicle (0.1% ethanol) was given in place of drinking water (Juan et al., 2002). Resveratrol consumption and the body weight gain were quantified throughout treatment in order to calculate and maintain the correct dose of resveratrol, which was based in the volume ingested and body weight.

2.4 Behavioral Testing

2.4.1Locomotor activity in open field test

In order to evaluate the effect of treatment with fluphenazine and/or resveratrol on spontaneous locomotor activity, the animals were placed individually in the center of an open field arena (60 cm of diameter) with black plywood walls and a white floor divided into 13 parts (Busanello et al., 2012; Röpke et al., 2014). The number of lines crossed and the number of rearing was measured over 5 min after 18 weeks of treatment.

2.4.2 Vacuous chewing movements (VCMs) quantification

VCMs were quantified after 18 weeks of fluphenazine and/or resveratrol treatment. The animals were individually placed in glass cages (20×20×19 cm), and after 6-min of habituation period, the number of VCMs was counted for additional six minutes as previously described (Fachinetto et al., 2007a, b; Busanello et al., 2012; Reis et al., 2013). VCMs were defined as single mouth openings on the vertical plane and not directed toward physical material. During the observation sessions, mirrors were placed under the floor of the experimental cage to permit observation when the animal was faced away from the observer. Experimenters were always blind to the treatments.

2.5 Ex vivo analyze

After behavioral tests, rats were euthanized by decapitation. Cortex and striatum were immediately dissected and stored at -80°C for biochemical analysis.

2.5.1 MAO activity

MAO activity was determined by measuring the kynuramine oxidation to 4-hydroxiquinoline (Villarinho et al., 2012; Reis et al., 2014; Oliveira et al., 2015). Brain homogenates of cortex and striatum (0.25 mg) were pre-incubated during 10 min at 37ºC with MAO-A (chlorgiline, 250 nM) or MAO-B (pargyline, 250 nM) inhibitors. Afterwards, kynuramine was added as MAO substrate in sub maximal concentrations (90 µM for MAO-A and 60 µM for MAO-B). Reaction medium was incubated for 30 min at 37ºC. After, the reaction was stopped with 10% trichloroacetic acid (TCA). The samples were centrifuged at 3.000*x g* for 5 min and the supernatant was used to estimate MAO activity. It was added 1 ml of 1N NaOH with an equal volume of supernatant. Product of reaction was measured spectrofluorimetrically at 488 nm for excitation and 520 nm for emission. Results were represented as nmol of 4-hydroxiquinoline/mg of protein/min.

2.5.2 Protein quantification

Protein content from samples was determined as described by Lowry et al. (1951), using serum bovine albumin as standard.

2.6 Statistical analysis

Data were analyzed by one-way, two-way ANOVA followed by *post hoc* test when appropriated. Pearson's correlation test was applied to verify a possible correlation between VCMs number and MAO activity. Significance was considered when $p < 0.05$.

3 Results

3.1 Effects of resveratrol and/or fluphenazine on body weight gain

Fluphenazine treatment caused a significant reduction on body weight gain ($F(3,26)=5.59$ and $p<0.05$) which was not prevented by resveratrol (Figure 1). Resveratrol *per se* did not alter the body weight gain of animals.

3.2Effects of chronic treatment with fluphenazine and/or resveratrol on locomotor and exploratory activity in rats

Fluphenazine administration caused a significant decrease in both locomotor ($F(3,26)=25.17$ and $p<0.05$; Figure 2A) and exploratory ($F(3,26)=28.82$ and $p<0.05$; Figure 2B) activity, represented by number of crossings and rearings in the open field test, respectively. Resveratrol treatment was not able to prevent the reduction neither in locomotor nor exploratory

activity caused by fluphenazine. Moreover, crossing or rearing numbers were not modified in the group treated with resveratrol alone (Figure 2).

3.3 Effects of fluphenazine and/or resveratrol on VCM

Chronic treatment (18 weeks) with fluphenazine increased the intensity of VCMs when compared with its vehicle ($F(3, 26)=10.04$ and $p<0.05$; Figure 3). The increase in VCMs was significantly reduced by co-treatment with resveratrol ($p<0.05$; Figure 3). Resveratrol *per se* did not induce VCMs when compared to the control (Figure 3).

3.4 Effects of chronic treatment with fluphenazine and/or resveratrol on MAO-A and MAO-B activity

The activities of MAO-A or MAO-B from cortex were not modified after chronic treatment with fluphenazine and/or resveratrol (Figure 4A and 4B). The activity of MAO-A in striatum was also not alter by treatments (Figure 4C). However, fluphenazine caused a significant reduction in striatal MAO-B activity ($F(3,26)=3.46$ and $p<0.05$) when compared to the resveratrol group (Figure 4D). A negative correlation was found between VCMs number and MAO-B activity in striatum [$r = -0.38$, $p = 0<0.05$] (Figure 5).

4. Discussion

TD is the most serious side effect provoked by long-term use of typical antipsychotics in humans. Although there are a large number of researchers investigating the possible mechanisms involved in the development of TD, the pathophysiology of disease as well as possibilities for effective treatments are still considered unknown (Andreassen and Jørgensen, 2000; Lister et al., 2014). In the present work we found that the VCMs induced by fluphenazine were reduced in rats treated with resveratrol and that there is a negative correlation between the number of VCMs and striatal MAO-B activity.

As previously mentioned, the pathophisiology of TD involves different neurotransmitter and receptor types (Gunne et al., 1984; Lee et al., 1997; Röpke et al., 2014; Lohr et al., 2003; Andreassen and Jørgensen, 2000). However, classical and actual studies continue investigating dopaminergic system as central in development of TD in humans (Rizos et al., 2010) and OD in animals (Fachinetto et al., 2007a, b; Freitas et al., 2015), exploring other points of this system beyond D_2 supersensitivity (Crane, 1973; Glazer et al., 1990; Andreassen and Jørgensen, 2000). Our group have demonstrated that experimental animals with high number of VCMs present a reduction in striatal dopamine uptake after chronic treatment with haloperidol or fluphenazine

(Fachinetto et al., 2007a, b). In this way, a recent study showed that the depletion of monoamines by reserpine causes motor injury in *Caenorhabditis elegans* due to dopaminergic alterations (Reckziegel et al., 2015). Of particular importance, in humans, one case report revealed that the improvement of DT symptoms was associated with an increase of TDA levels in striatum (Rizos et al., 2010). Furthermore, the activation of CB1 receptors, which indirectly regulate the release of dopamine, decreased the VCMs induced by haloperidol in rats (Röpke et al., 2014).

In this scenario, it is important consider the protective action of resveratrol against the dopaminergic neurotoxicity in experimental animals (Blanchet et al., 2008; Jin et al., 2008). Our group found that the resveratrol in low doses (1 and 5 mg/kg) is able to reduce VCMs induced by reserpine in mice (Busanello et al., 2011) or by fluphenazine in rats in an acute model (Busanello et al., 2012). These data suggest that resveratrol acts in both models through a common mechanism. However, the action mechanism of resveratrol as well as its effect on VCMs in a chronic model of OD induced by fluphenazine was not investigated. It has been previously demonstrated that some substances with promissory effects on acute models of OD does not exhibit the same efficacy on chronic models (Burger et al., 2006; Fachinetto et al., 2007a, b; Pereira et al., 2011). Considering such aspects, our first objective was to investigate the effects of resveratrol on VCMs in rats chronically treated with fluphenazine, using a dose of 20 mg/kg by oral route since this is the way that population consumes resveratrol in the food. The body weight gain of the animals was used as an indicative of toxicity, since we utilized a higher dose of resveratrol (20 mg/kg) in relation to our previous studies and that there are few studies evaluating chronic administration of resveratrol. In the literature it is possible found studies using a wide range of resveratrol doses, varying from 1 to 100 mg/kg (Zhang et al., 2010). It is important to emphasize that there is no safe dose established to humans for the use of resveratrol.

In agreement with previous data from our group, fluphenazine significantly reduced weight gain of animals (Fachinetto et al., 2005, 2007b) and the co-treatment with resveratrol or resveratrol alone did not modify this parameter. Regarding VCMs, the numbers were increased by chronic treatment with fluphenazine, and the co-treatment with resveratrol reduced the intensity of them. As previously demonstrated, fluphenazine administration caused a decrease in locomotor and exploratory activity (Fachinetto et al., 2007a; Busanello et al., 2012). However, co-treatment with resveratrol did not cause any change in these parameters. Similar responses were observed in acute models using reserpine (Busanello et al., 2011) and fluphenazine (Busanello et al., 2012). These data suggest that resveratrol exhibits a similar

effect in acute and chronic models. Furthermore, the fact of compound is able to reduce VCMs without altering locomotor and exploratory activity reinforce the idea that it might be acting differentially considering anatomical locals since VCMs have been linked to ventrolateral neostriatum area, whereas the suppression of locomotion resulting from dopamine depletion to the nucleus accumbens (Kelley et al., 1989; Salamone et al., 1998).

Our second objective was to investigate the role of MAO activity in the action of resveratrol on VCMs and/or in the VCMs development, since some studies have pointed the participation of enzyme in these events (Andreassen and Jørgensen, 2000; Freitas et al., 2015). It is known that pharmacological effects are explored to the inhibition of MAO-B for anti-parkinsonian drugs (Marconi et al., 2014) while the inhibition of MAO-A promotes anti-apoptotic and antidepressant effects (Youdim and Bakhle, 2006; Youdim et al., 2006; Shuto et al., 2013). Regarding resveratrol, there is evidence that *cis*- and *trans*-resveratrol were able to reduce noradrenaline and 5-hydroxytryptamine uptake and inhibit the activity of both isoforms of MAO (Yáñez et al., 2006). Here, no difference was found in MAO activity among groups. The particular absence of effect of resveratrol on MAO activity might be related with the route of administration, that via metabolism could modify the structure of compound, difficulting its interaction with the enzyme (Erdogan and Vang, 2016) since there is evidence that resveratrol inhibits MAO *in vitro* (Yáñez et al., 2006). Indeed, resveratrol inhibits MAO in a reversible way to MAO-B and partially reversible to MAO-A (Busanello et al., unpublished data) which could difficult the inhibition in ex vivo analyses.

An important effect found in the present study was the relation of VCMs with the activity of MAO-B from striatum. There was a decrease in MAO-B activity in those rats that presented the highest number of VCMs, which was visualized by negative correlation found among parameters. In accordance, a recent study from our group demonstrated that the VCMs induced by reserpine in mice were related with a reduction in MAO activity (Freitas et al., 2015), pointing the important role of MAO in development of VCMs in animal models. As acute administration of antipsychotics blocks dopamine receptors blockage and increase dopamine synthesis as well as its metabolism by MAO (Lohr, 1991; Andreasen and Jørgensen, 2000) with consequent reactive species generation (Youdim and Bakhle, 2006), we suppose that chronically, the MAO enzyme, which is sensible to oxidative stress, had a decrease in its function associated with VCMs maintenance. However, despite of antioxidant properties of resveratrol, it did not protect all animals of development of OD, a phenomenon previously observed with other antioxidant substances (Fachinetto et al., 2007a; Busanello et al., 2012; Peroza et al., 2013).

5. Conclusion

In conclusion, resveratrol seems to be a promissory molecule to treat chronic OD. Furthermore, we can suggest that reduction in MAO-B activity is associated to chronic VCMs. Future studies are necessary to investigate the mechanisms involved in the modulation of MAO activity in OD model.

Acknowledgments

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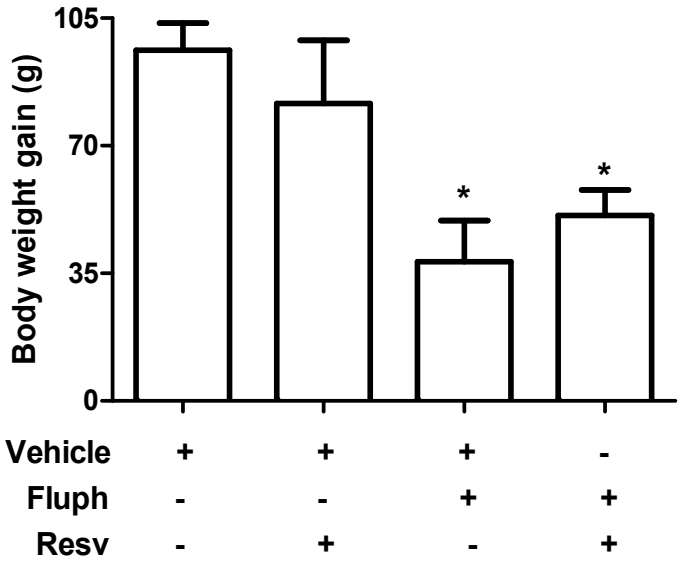

Figure 1: Effect of chronic treatment (18 weeks) with resveratrol (20 mg/kg, in drinking water, every day) and/or fluphenazine (25 mg/kg, i.m., each 21 days) on body weight gain in rats. Values represent mean±S.E.M.; vehicle, n=5; resveratrol, n=5; fluphenazine, n=9; fluphenazine+resveratrol, n=8. Data were analyzed by one-way ANOVA followed by Tukey's test. *Significant difference from vehicle and resveratrol groups.

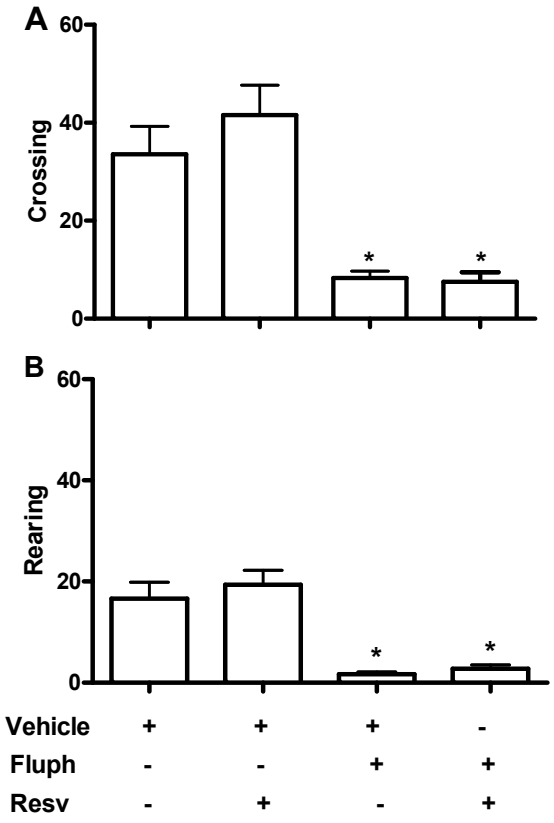

Figure 2: Effects chronic of resveratrol (20 mg/kg, in drinking water, every day) and/or fluphenazine (25 mg/kg, i.m., each 21 days) on number of (A) crossing and (B) rearing in open field test. Values are represented as mean±S.E.M.; vehicle, n=5; resveratrol, n=5; fluphenazine, n=9; fluphenazine+resveratrol, n=8. Data were analyzed by two-way ANOVA followed by Tukey's test. *Significant difference from vehicle and resveratrol group.

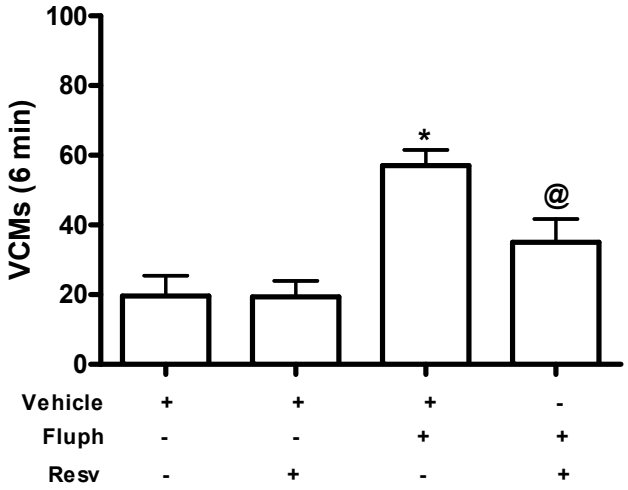

Figure 3: Effect of chronic treatment (18 weeks) with resveratrol (20 mg/kg, in drinking water, every day) on fluphenazine (25 mg/kg, i.m., each 21 days)-induced VCMs in rats. Values represent mean±S.E.M.; vehicle, n=5; resveratrol, n=5; fluphenazine, n=9; fluphenazine+resveratrol, n=8. Data were analyzed by one-way ANOVA followed by Tukey's test. *Significant difference from vehicle and resveratrol groups. @ Significant difference from fluphenazine group.

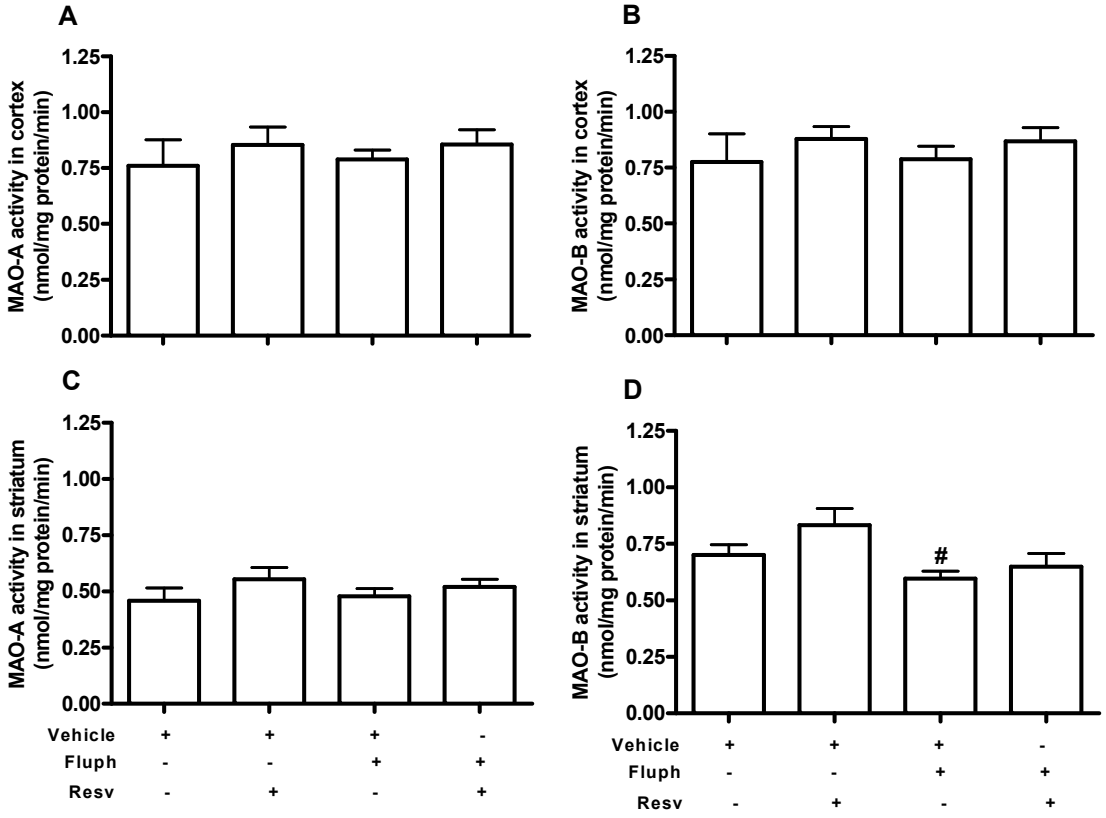

Figure 4: Effects of chronic treatment (18 weeks) with resveratrol (20 mg/kg, drinking water, every day) and/or fluphenazine (25 mg/kg, i.m., each 21 days) on MAO-A (A, C) and MAO-B (B, D) activity in cortex (A, B) and striatum (C, D) of rats. Values are expressed as means ± S.E.M.; vehicle, n=5; resveratrol, n=5; fluphenazine, n=9; fluphenazine+resveratrol, n=8. Data were analyzed by one-way ANOVA followed by Tukey's test. *Significant difference from vehicle group.

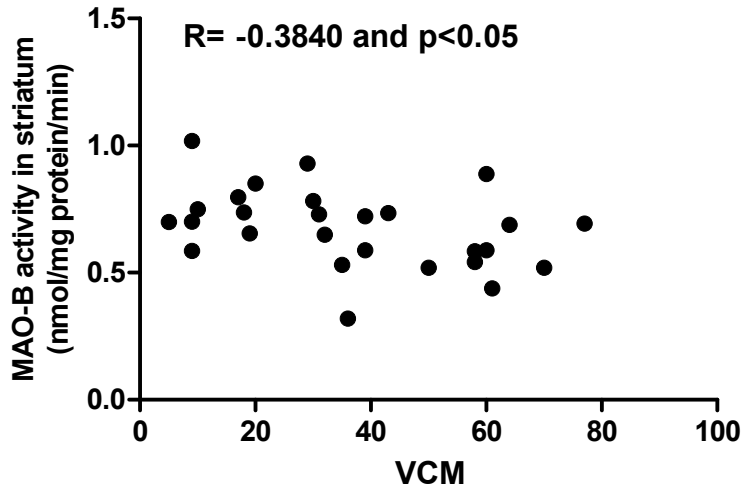

Figure 5: Correlation between VCMs number and MAO-B activity in striatum of rats. Significant correlations (Pearson correlation – r) when $p < 0.05$

4. DISCUSSÃO

A DT é considerada o efeito colateral mais grave associado ao uso prolongado de antipsicóticos típicos, acometendo um alto percentual de pacientes que fazem uso crônico destes fármacos. Apesar da grande quantidade de trabalhos propondo mecanismos envolvidos no desenvolvimento da DT, sua patofisiologia bem como possibilidades de tratamento efetivos ainda são considerados como incógnitas para estudiosos da área (Andreassen & Jørgensen, 2000).

Os mecanismos precisos envolvidos no desenvolvimento da DT continuam não elucidados, mas parecem envolver múltiplos sistemas de neurotransmissores e tipos de receptores (Para revisão ver, Andreassen e Jørgensen, 2000; Gunne et al., 1984; Lee et al., 1997; Lohr et al., 2003). Inicialmente, a supersensibilidade dos receptores de DA D_2 foi proposta como a principal base para explicar a DT, hipótese esta suportada por trabalhos mostrando um aumento na densidade de receptores D_2 no estriado de ratos após administração aguda (2 a 3 semanas) de antipsicóticos, incluindo a flufenazina (Burt et al., 1977; Boyson et al., 1988). Contudo, outros estudos não têm encontrado alterações nos receptores estriatais D_2 após tratamento crônico (18 semanas) de ratos com flufenazina (Van Kampen & Stoessl, 2000). Além disso, existe uma pobre correlação temporal e espacial entre o desenvolvimento da DT e a supersensibilidade dos receptores para DA (Ver para revisão, Gunne et al., 1984; Gerlach & Casey, 1988). Dados da literatura sugerem que outros componentes do sistema dopaminérgico se encontram alterados após tratamento com antipsicóticos e estão relacionados à DT em humanos e DO em animais. Podemos citar como exemplo, alterações no TDA, onde estudos de nosso grupo demonstraram que animais experimentais com alto número de MMV apresentaram uma redução na captação de DA no estriado após tratamento crônico com haloperidol ou flufenazina (Fachinetto et al., 2007a; 2007b). Corroborando com esta idéia, um relato de caso em humanos demonstrou que a melhora dos sintomas da DT estava associada com um aumento nos níveis estriatais do TDA (Rizos et al., 2010).

Com relação a MAO, principal enzima envolvida no metabolismo da DA, dados da literatura indicam que a administração de antipsicóticos, por bloquear receptores dopaminérgicos, poderia causar um aumento secundário na síntese de dopamina e, consequentemente, um aumento no seu metabolismo (Lohr, 1991; Andreasen e Jørgensen, 2000). Este aumento na metabolização da DA, por oxidases, forma como produto o peróxido de hidrogênio (H_2O_2) que ao reagir com metais de transição via reação de fenton, forma radicais livres, como radicais hidroxila ($OH^{\cdot}$) e superóxido. Além disso, a própria DA pode sofrer auto-oxidação formando dopamina quinona que pode agir como espécie reativa de oxigênio (Lohr,

1991; 2003) ocasionando o estresse oxidativo. Este por sua vez, também sugerido na literatura como um importante evento na patogênese da DT e DO (Andreassen & Jørgensen, 2000). Desta forma, substâncias com propriedades antioxidantes (Reis et al., 2013; Peroza et al.,2013) e com potencial para inibir a MAO, como é o caso da selegilina (Sachdev et al., 1999) apresentaram relativa eficácia em modelos experimentais de DO.

Neste contexto, tem sido demonstrado que o resveratrol possui ação protetora contra a neurotoxicidade dopaminérgica em animais de experimentação (Jin et al., 2008; Blanchet et al., 2008), incluindo modelo de parkinsonismo induzido por reserpina (Busanello et al., 2011). No entanto, não existem relatos na literatura demonstrando se o resveratrol reduz as alterações observadas em modelos de DO induzida por antipsicóticos. Com isto, o primeiro objetivo deste estudo foi investigar os efeitos do resveratrol (utilizando uma dose baixa), em um modelo agudo de MMV induzido pela administração de flufenazina em ratos. Neste trabalho, foi observado que o resveratrol, na dose de 1 mg/kg administrado 3 vezes na semana, reduziu a prevalência dos MMV, mas não a intensidade da DO, representada pelo número de MMV. Recentemente, nosso grupo demonstrou que o resveratrol impediu também o desenvolvimento de MMV em um modelo animal de parkinsonismo induzido por reserpina em camundongos (Busanello et al., 2011). Este efeito do resveratrol em atenuar o MMV em ambos os modelos de DO sugere que o mesmo provavelmente atue em pontos comuns à ambos os modelos, diferentemente do que ocorreu quando a *Valeriana officinallis* foi testada, a qual foi eficaz somente em reverter os MMV induzidos por reserpina (Pereira et al., 2011) e não por haloperidol em ratos (Fachinetto et al., 2007b).

Foi anteriormente publicado que o tratamento com *cis*- e *trans*-resveratrol reduziu a captação de noradrenalina e 5-hidroxitriptamina e também a atividade da MAO (Yáñez et al., 2006); o que, consequentemente, poderia aumentar a atividade locomotora e exploratória em roedores. Assim, nós também avaliamos neste trabalho o desempenho locomotor e exploratório dos animais no teste de campo aberto. Sabe-se que o bloqueio de receptores D_2 e em menor grau os receptores D_1 causa uma diminuição na locomoção e no comportamento exploratório (Redrobe et al., 1998). Como esperado, foi encontrado uma redução na atividade locomotora e exploratória nos animais tratados agudamente com flufenazina. No entanto, o tratamento com resveratrol não protegeu completamente os animais da hipolocoloção induzida pela administração de flufenazina. De acordo, Busanello et al. (2011), o tratamento com resveratrol não evitou a hipolocomoção causada pela reserpina em camundongos. Esses resultados comportamentais divergentes podem estar associados a um possível efeito do resveratrol tecido específico, uma vez que os MMV estão relacionados a alterações neuroquímicas

preferencialmente no neoestriado ventrolateral enquanto que a supressão da locomoção com a depleção de DA no nucleo acumbens (Kelley et al., 1989; Salamone et al., 1998). Além disso, a neuroproteção exibida pelo resveratrol em nosso protocolo experimental pode estar relacionada com as propriedades antioxidantes do resveratrol, uma vez que existem evidências na literatura mostrando que o pré-tratamento com resveratrol reduz o estresse oxidativo e a apoptose causada pela degradação da dopamina em células SH-SY5Y (Lee et al., 2007). Somando-se a isso, dados da literatura demonstram que o resveratrol inibe tanto a MAO-A quanto a MAO-B (Shuto et al., 2013). A inibição da MAO-B já é muito explorada para fármacos antiparkinsonianos (Marconi et al., 2014) enquanto que a inibição da MAO-A promove efeitos antiapoptóticos (Shuto et al., 2013).

De fato, foi demonstrado que o *cis*- e o *trans*-resveratrol reduziram a captação de noradrenalina e 5-hidroxitriptamina e inibiram a atividade de ambas isoformas da MAO (Yáñez et al., 2006). Contudo, Zohu et al., 2001 demonstraram que o resveratrol inibe somente a atividade da MAO-A em ratos. Considerando estes dados contraditórios, avaliamos o potencial inibitório do resveratrol sobre a atividade da MAO, determinamos parâmetros cinéticos e a possível reversibilidade da reação. O Resveratrol inibiu ambas isoformas da MAO com uma potência aproximada de 28 vezes maior para a MAO-A do que para a MAO-B. Estes dados estão de acordo com um estudo prévio que demonstrou que o resveratrol inibe a MAO-A em uma concentração menor do que para a MAO-B (Yáñez et al., 2006). O resveratrol não alterou os valores de K_m para ambas isoformas da MAO. Contudo, ele causou uma diminuição nos valores de V_{max} para MAO-A e MAO-B comparadas aos valores de V_{max} obtidos na ausência de resveratrol para todas as concentrações testadas, sugerindo que uma interação esteja ocorrendo em um local diferente do sítio ativo caracterizando uma inibição não competitiva. Após 24 horas de diálise, a atividade da MAO-A foi recuperada parcialmente, sendo dependente da concentração de resveratrol utilizada. Com relação a MAO-B, a atividade retornou a valores superiores a 80% caracterizando uma inibição completamente reversível (Harfenist et al., 1996). Considerando estes dados, é possível sugerir que parte dos efeitos neuroprotetores do resveratrol são devido a habilidade em inibir a atividade da MAO e consequentemente o estresse oxidativo.

Com o objetivo de entender como os isômeros do resveratrol interagem com a MAO e causam a sua inibição, usamos o programa Vina para ampliar o estudo usando um modelo com a estrutura da MAO humana cristalizada. De acordo com os resultados do docking para MAO-A e MAO-B, em ambos os casos os isômeros do resveratrol interagem em regiões distintas das proteínas. O *cis*-resveratrol interage através de ligações de hidrogênio no sítio ativo da MAO-

A, próximo ao local de ligação do cofactor Flavina adenina dinucleotideo (FAD). O *trans*-resveratrol ligou-se em outra região, próxima a região C-terminal da MAO-A ligada a membrana (Wang et al., 2013), através de uma interação cation-π com a Arg129, um arranjo π-π com a His488, e uma ligação de hidrogênio com a Asn125. Com a MAO-B, o oposto ocorreu, o *trans*-resveratrol interagiu no sítio do cristal ligante, fazendo um arranjo π-π com Tyr398 e Tyr435, e uma ligação de hidrogênio com o nitrogênio do FAD. A presença do arranjo π-π na Tyr398 e Tyr435 demonstra sua importância na estabilização do ligante no sítio ativo através de interações hidrofóbicas, similar aos resultados demonstrados por Mustafa Toprakçí and Kemal Yelekçi (2005), Simona Distinto et al (2012) e Kare et al (2013). Por outro lado, o *cis*-resveratrol, liga-se a uma região equivalente da MAO-A próxima a região C-terminal da proteína, interagindo com os resíduos Asp123 e Asn116 através de ligações de hidrogênio. Os valores de ΔG obtidos demonstram que na MAO-A ambos isômeros do resveratrol interagem praticamente com a mesma afinidade, mas na MAO-B o *trans*-resveratrol apresenta uma afinidade maior quando comparada com o *cis*-resveratrol. Em 2013, Chi Chiu Wang et al. demonstraram que ambas MAO-A e B apresentam 73% dos resíduos conservados. Esta pequena diferença pode estar envolvida nas formas distintas dos isômeros do resveratrol interagirem com as enzimas.

As diferenças encontradas no provável local de ligação *in vitro* e *in silico* podem ser devido às diferenças entre a MAO-A de rato e MAO-A humana. É conhecido que uma das principais diferenças entre ambas isoformas da enzima é a forma e a configuração dos seus respectivos sítios de ligação ao substrato. Uma diferença significativa nas estruturas da MAO-A humana e de rato é a conformação da alça que forma a cavidade formada pelos resíduos 210–216 (De Colibus et al., 2005). Na MAO-A de rato, esta alça é o contrário do que a encontrada em humanos, embora esta alça seja homologa na MAO-B humana. Esta diferença é responsável pelo menor volume da cavidade do sítio ativo da MAO-A de rato (Youdim et al., 2006; Edmondson et al., 2009).

Sabe-se que existem controvérsias na literatura em relação aos modelos utilizados para o estudo da DT. Modelos agudos de DO, embora bem aceitos, têm sido criticados por alguns autores e classificados como Parkinsonismo ou síndrome extrapiramidal (SEP), porque normalmente a reversão é possível descontinuando o uso do antipsicótico ou através da administração de fármacos anticolinérgicos (Egan et al., 1996; Andreassen e Jørgensen 2000). Já modelos crônicos são os que apresentam uma maior similaridade de respostas com a DT, pois são de difícil reversão e, em geral, os MMV são exacerbados após a retirada do antipsicótico (Andreassen e Jørgensen 2000). Considerando tais aspectos, o terceiro objetivo

deste trabalho foi investigar o efeito do resveratrol (administrado por via oral) em um modelo crônico de DO induzida por flufenazina em ratos, bem como avaliar se a alteração na atividade da MAO poderiam estar envolvidos no possível efeito protetor do resveratrol e/ou como parâmetro associado ao desenvolvimento dos MMV. Foi analisado primeiramente o ganho de peso dos animais ao longo das 18 semanas de tratamento como indicativo de toxicidade, uma vez que usamos, neste protocolo, uma dose maior de resveratrol (20 mg/kg) e por via oral. Além disso, são escassos os trabalhos que avaliam de forma crônica o uso de resveratrol. É importante enfatizar que para humanos não há dose segura estabelecida para o uso do resveratrol de forma isolada. Em acordo com dados prévios de nosso grupo, a flufenazina reduziu de forma significante o ganho de peso dos animais (Fachinetto et al., 2005; 2007b) e o co-tratamento com resveratrol não alterou este efeito. Além disso, não houve alteração no ganho de peso dos animais tratados com resveratrol em relação ao grupo controle. Com relação aos MMV, o tratamento crônico com flufenazina aumentou o número de MMV enquanto que o co-tratamento com resveratrol também reduziu a intensidade dos MMV. A administração de flufenazina causou uma diminuição na atividade locomotora e exploratória. No entanto, a administração concomitante de resveratrol não promoveu qualquer alteração neste quadro. Esses dados vão ao encontro dos resultados encontrados em modelos agudos com reserpina em camundongos e flufenazina em ratos (Busanello et. al., 2011 e 2012) sugerindo que o resveratrol parece ter um efeito semelhante em modelos agudos e crônico e para ambas as doses testadas. De fato, na literatura encontramos uma ampla variedade de doses de resveratrol sendo utilizadas, desde 1 mg/kg até 100 mg/kg (Zhang et al., 2010).

No que se refere à atividade da MAO, não observamos diferença entre os grupos quando a estrutura cerebral analisada foi o córtex. Apenas a atividade da MAO-B no estriado dos grupos tratados com flufenazina foram diferentes do grupo resveratrol. Tendo em vista que outros autores encontraram alteração na atividade desta enzima *in vitro* (Yáñez et al., 2006) e em nosso estudo, nenhum efeito significante foi observado, acreditamos que a ligação do resveratrol por se dar de forma parcialmente ou totalmente reversível dificultou a observação de qualquer efeito *ex vivo*. Além disso, sabe-se que o resveratrol possui vários alvos celulares que podem estar contribuindo para o efeito protetor do mesmo na DO independente da inibição da MAO (Virmani et al., 2013).

De particular importância, foi observado que os MMV parecem estar associados com a inibição da MAO-B no estriado demonstada pela correlação negativa encontrada entre ambos os parâmetros. De acordo, um resultado semelhante foi demonstrado por nosso grupo onde o aumento nos MMV induzidos por reserpina em camundongos foi relacionado à diminuição na

atividade da MAO (Freitas et al. 2015) sugeriendo que esta enzima possui um papel importante no desenvolvimento dos MMV em modelos animais.

5. CONCLUSÕES ESPECÍFICAS

- O resveratrol reduziu a prevalência da DO induzida por tratamento agudo com flufenazina;
- *In vitro*, o resveratrol causa inibição não competitiva sobre a MAO, através de ligação parcialmente reversível para a MAO-A e totalmente reversível para a MAO-B e *in silico*, o resveratrol interage com o sítio ativo da MAO;
- O tratamento crônico com resveratrol, por via oral, diminuiu a intensidade da DO induzida por flufenazina, efeitos estes que não parecem estar relacionados com alterações na atividade da MAO.

6. CONCLUSÃO GERAL

De maneira geral podemos concluir que as doses de resveratrol usadas neste estudo foram eficazes em reduzir a DO em ratos e que provavelmente os efeitos protetores exibidos pelo composto não sejam dependentes de sua ação direta sobre a MAO. Desta forma, podemos sugerir que o resveratrol parece ser um agente neuroprotetor promissor como terapia adjuvante no tratamento da DT em humanos.

7. PERSPECTIVAS

- Quantificar a biodisponibilidade plasmática do resveratrol nos diferentes protocolos experimentais;

- Investigar, em ratos tratados com flufenazina e/ou resveratrol, possíveis alterações no TDA, tirosina hidroxilase e NRF-2.

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9. ANEXO A: CARTA DE APROVAÇÃO DO COMITE DE ETICA 051/20011

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMITÊ INTERNO DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL-UFSM

CARTA DE APROVAÇÃO

O Comitê Interno de Ética em Experimentação Animal-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Efeito do resveratrol em modelos de parkinsonismo e discinesia induzidos por neurolépticos em ratos"

Numero do Parecer: 051/2011

Pesquisador Responsável: Roselei Fachinetto

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

Os membros da CIETEA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO:

Santa Maria, 9 de Maio de 2011.

Marta Lizandra do Rêgo Leal
Coordenador do Comitê Interno de Ética em Experimentação
Animal-UFSM

10. ANEXO B – CARTA DE APROVAÇÃO DO COMITE DE ÉTICA 091/2013

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Efeito do resveratrol nas alterações comportamentais, bioquímicas e doparminérgicos induzidos por flufenazina em ratos: possível influência do fator gênero."

Número do Parecer: 091/2013

Pesquisador Responsável: Profa. Dra. Roselei Fachinetto

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO: 12/12/2013.

Santa Maria, 12 de dezembro de 2013.

Prof. Dr. Alexandre Krause
Coordenador da Comissão de Ética no Uso de Animais- UFSM

Comissão de Ética no Uso de Animais - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362