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ORIGINAL PAPER

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 such as tardive dyskinesia (TD) in humans and orofacial dyskinesia (OD) in rodents. The disease mechanisms as well as treatment effectiveness are still unknown. 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 relationship between monoamine oxidase (MAO) activity and vacuous chewing movements (VCMs). Rats were treated for 18 weeks with fluphenazine enantate [25 mg/kg, intramuscularly (i.m.), every 21 days] and/or resveratrol (20 mg/kg, offered daily in drinking water). Next, body weight gain, behavioral parameters (VCMs and open field tests—locomotor and rearing activity), and MAO activity were evaluated. Fluphenazine treatment reduced body weight gain, number of crossings and rearings, and the co-treatment with resveratrol did not affect these alterations. Fluphenazine increased the prevalence and intensity of VCMs and the co-treatment with resveratrol reduced the VCMs. Furthermore, a negative correlation was found between the number of VCMs and MAO-B activity in the striatum of rats. Our data suggest that resveratrol could be promissory to decrease OD. Moreover, MAO-B activity in the striatum seems to be related to VCMs intensity.

Keywords Tardive dyskinesia · Orofacial dyskinesia · Resveratrol · MAO enzyme

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Introduction

Schizophrenia is one of the most debilitating psychiatric diseases which affects ~1% of the population [1, 2]. The complexity of this disease makes the treatment quite difficult, with typical and atypical antipsychotics being the most effective drugs used currently [3]. The pharmacological mechanism of typical antipsychotics, such as fluphenazine, involves the blockage of dopamine D_2 receptors in the mesolimbic area [4–6]. However, they also act on the nigrostriatal pathway causing debilitating motor effects such as tardive dyskinesia (TD) [7].

TD is characterized by hyperkinetic, repetitive, and involuntary movements of the orofacial region, also affecting the neck, limbs (especially the upper limbs) and trunk [8–10]. It may occur during or after the discontinuation of chronic treatment with antipsychotics and might be irreversible even after antipsychotic withdrawal [10]. Some researchers estimate that the prevalence of TD in patients receiving treatment with typical antipsychotics is ~20–25% [11], which increases with age and affects ~50% of patients older than 50 years of age [12–14]. Although there are many researchers investigating the mechanisms involved in TD and/or possible treatments, little 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 the development of TD in humans and orofacial dyskinesia (OD) represented by vacuous chewing movements (VCMs) in rodents [15]. In this scenario, some researchers have highlighted the role of the enzyme monoamine oxidase (MAO) [16, 17]. MAO participates in the metabolism of monoamines, including dopamine (DA), norepinephrine and serotonin [18]. Literature reports show that the administration of antipsychotics and the consequent blockage of dopamine receptors increases dopamine synthesis and its metabolism by MAO [10, 16]. 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·) and anion superoxide (O^-_2) radicals [19]. In addition, DA can itself undergo autoxidation to form quinones of dopamine, which are potent oxidant species [16, 17]. 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 this enzyme in OD [20]. On the other hand, a few reporters point to a relative effectiveness of MAO inhibitors in experimental models of OD [21].

Resveratrol is a phytoalexin found in grapes, cranberries, and peanuts [22] which exhibits antioxidant properties. In addition, resveratrol has several pharmacological effects, including neuroprotection [23–26]. Of particular importance to our study, there is evidence that resveratrol may modulate some proteins of the dopaminergic system including MAO [27, 28]. Furthermore, our group recently showed that acute exposure to low doses decreases both VCMs induced by reserpine in mice [29] and VCMs induced by fluphenazine in rats [30]. However, studies have demonstrated that some substances that have promissory effects on acute models of OD do not have the same efficacy on chronic models [31–35]. Chronic models of OD present more similarities with TD than acute models which are characterized as extrapyramidal syndrome [10].

Data about the role of resveratrol against VCMs induced by chronic treatment with fluphenazine and the participation of MAO is not available for this model. Considering these 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.

Materials and Methods

Animals

Adult male Wistar rats (60 days old), weighing 200–220 g, were purchased from a breeding colony at 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 (number of 051/2011).

Drugs

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

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 enantate was carried out for 18 weeks [31]. Fluphenazine enantate (25 mg/kg) or its vehicle (soy oil, 1 mL/kg) was administered every 21 days intramuscularly (i.m.) [30, 31]. Concomitantly, resveratrol (20 mg/kg) or its vehicle (0.1% ethanol) was administered instead of drinking water [36]. Resveratrol consumption and body weight gain were quantified throughout the treatment to calculate and maintain the correct dose of resveratrol, which was based on the volume ingested and body weight.

Behavioral Testing

Locomotor Activity in Open Field Test To evaluate the effect of fluphenazine enantate and/or resveratrol treatment on the spontaneous locomotor activity, the animals were placed individually in the center of an open field arena (60 cm diameter) with black plywood walls and a white floor divided into 13 parts [30, 37, 38]. The number of lines crossed and the number of rearing was measured over 5 min after 18 weeks of treatment.

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 cm × 20 cm × 19 cm); after a 6-min of habituation period, the number of VCMs of each animal was counted for an additional 6 min as previously described [30–32, 38]. 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. It was

also verified the prevalence of VCMs in the since the administration of fluphenazine does not develop VCMs in all treated animals [39, 40]. Thus, the animals presenting more than 40 VCMs were considered as +VCM, as previously described [30, 31].

Ex Vivo Analysis After the behavioral tests, rats were euthanized by decapitation. The cortex and striatum were immediately dissected and stored at −80 °C for biochemical analysis.

MAO Activity MAO activity was determined by measuring kynuramine oxidation to 4-hydroxiquinoline [42–44]. Brain homogenates of the cortex and striatum (0.25 mg) were pre-incubated for 10 min at 37 °C with MAO-A (chlorgiline, 250 nM) or MAO-B (pargyline, 250 nM) inhibitors. Next, kynuramine was added as MAO substrate at sub-maximal concentrations (90 µM for MAO-A and 60 µM for MAO-B). Reaction medium was incubated for 30 min at 37 °C; the reaction was stopped with 10% trichloroacetic acid (TCA). The samples were centrifuged at 3.000*g* for 5 min, and the supernatant was used to estimate MAO activity. To an equal volume of supernatant, 1 mL of 1 N NaOH was added. The product of the reaction was measured using a spectrofluorometer at 488 nm for excitation and 520 nm for emission. The results were represented as nmol of 4-hydroxiquinoline/mg of protein/min.

Protein Quantification The protein content in the samples was determined as described by Lowry et al. [45], using serum bovine albumin as the standard.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey's test when appropriate. Data were shown as mean ± standard error of mean (SEM). The prevalence data was analyzed using Chi-square test. Pearson's correlation test was applied to verify a possible correlation between the number of VCMs and MAO activity in the striatum. Significance was set at $p < 0.05$. The number of animals was 5–9 per group.

Results

Effects of Resveratrol and/or Fluphenazine on Body Weight Gain

Fluphenazine enantate treatment caused a significant reduction on body weight gain [$F(3,26) = 5.59$ and $p < 0.05$] which was not prevented by resveratrol (Fig. 1). Resveratrol treatment alone did not alter the body weight gain in animals.

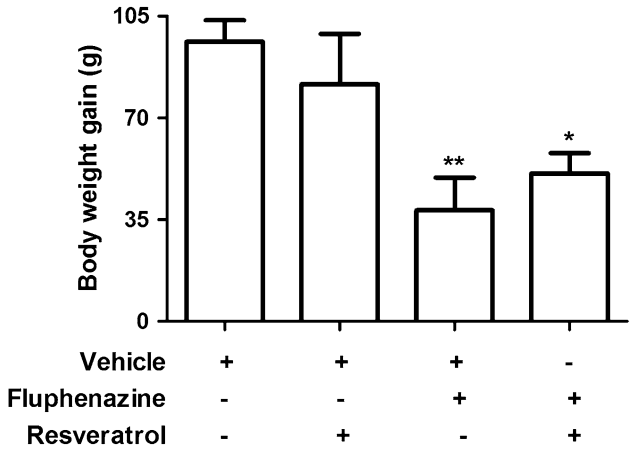

Fig. 1 Effect of chronic treatment (18 weeks) with resveratrol (20 mg/kg, in drinking water, every day) and/or fluphenazine enantate [25 mg/kg, intramuscularly (i.m.), every 21 days] on body weight gain in rats. Data are expressed as mean ± standard error of mean (SEM) (n = 5–9) *p < 0.05 and **p < 0.01 compared with vehicle group. (One-way analysis of variance (ANOVA) followed by Tukey's test)

Effects of Chronic Treatment with Fluphenazine and/or Resveratrol on Locomotor and Exploratory Activity in Rats

Fluphenazine enantate administration caused a significant decrease in both locomotor [$F(3,26) = 25.17$ and $p < 0.05$; Fig. 2a] and exploratory [$F(3,26) = 28.82$ and $p < 0.05$; Fig. 2b] activities, represented by the number of crossings and rearings in the open field test, respectively. Resveratrol treatment did not prevent the reduction neither in locomotor nor exploratory activity caused by fluphenazine enantate. Moreover, the crossing or rearing numbers were not modified in the group treated with resveratrol alone (Fig. 2).

Effects of Fluphenazine and/or Resveratrol on VCM

Chronic treatment (18 weeks) with fluphenazine enantate increased the intensity [$F(3,26) = 10.04$ and $p < 0.05$; Fig. 3] and prevalence (Chi-squared = 10.37 and $p < 0.05$; Fig. 4) of VCMs when compared with its vehicle. Co-treatment with resveratrol avoided the increase in intensity ($p < 0.05$; Fig. 3) and prevalence (Chi-squared = 7.14 and $p < 0.05$; Fig. 4) of VCMs. Resveratrol alone did not alter both parameters of VCMs (Figs. 3, 4).

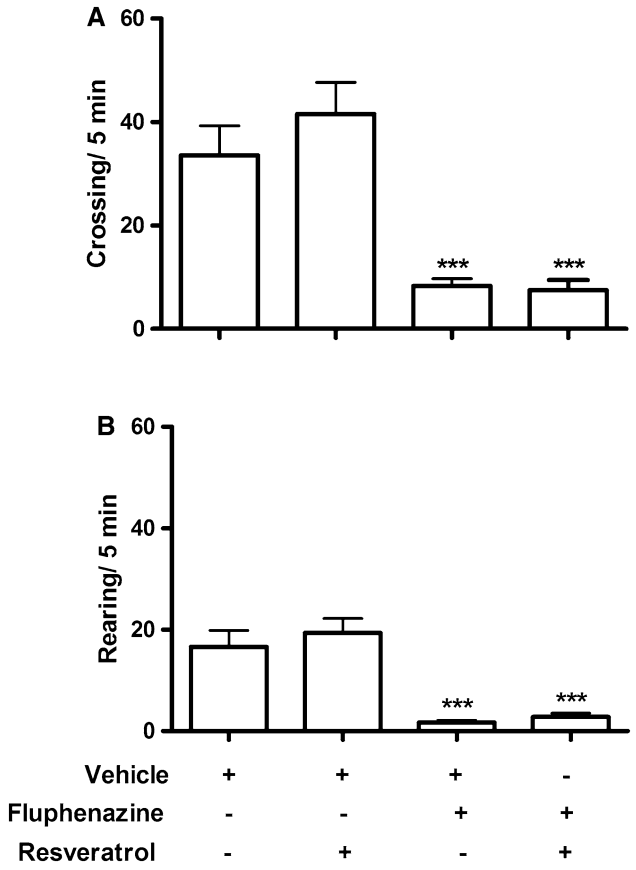

Fig. 2 Effects of chronic treatment with resveratrol (20 mg/kg, in drinking water, every day) and/or fluphenazine enantate [25 mg/kg, intramuscularly (i.m.), every 21 days] on the number of (**a**) crossing and (**b**) rearing in the open field test. Data are expressed as mean ± standard error of mean (SEM) (n = 5–9). ***p < 0.001 compared with vehicle and resveratrol groups [one-way analysis of variance (ANOVA) followed by Tukey's test]

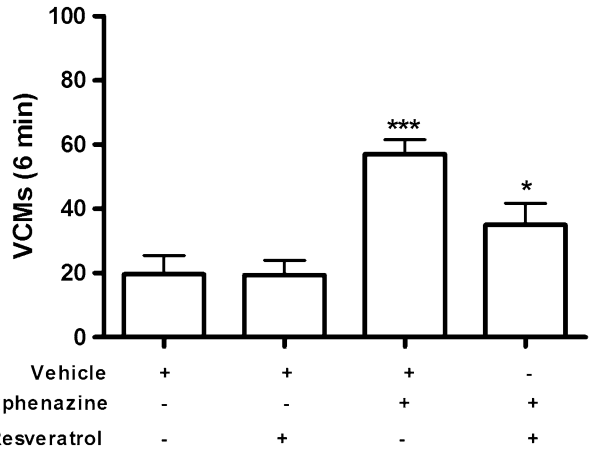

Fig. 3 Effect of chronic treatment (18 weeks) with resveratrol (20 mg/kg, in drinking water, every day) on the intensity of vacuous chewing movements (VCM) induced by fluphenazine enantate [25 mg/kg, intramuscularly (i.m.), every 21 days] in rats. Data are expressed as mean ± standard error of mean (SEM) (n = 5–9). *p < 0.05 compared with fluphenazine enantate treated group; ***p < 0.001 compared with vehicle and resveratrol groups [one-way analysis of variance (ANOVA) followed by Tukey's test]

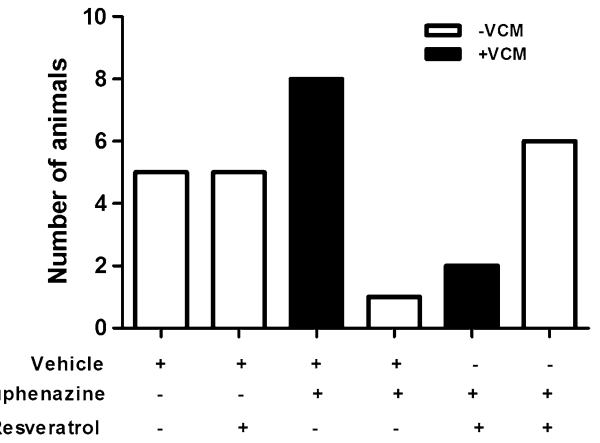

Fig. 4 Effect of chronic treatment (18 weeks) with resveratrol (20 mg/kg, in drinking water, every day) on the prevalence of vacuous chewing movements (VCM) induced by fluphenazine [25 mg/kg, intramuscularly (i.m.), every 21 days] in rats. Values represent the number of animals presenting more than 40 VCMs (+VCM) or less than 40 VCMs (−VCM). Data were analyzed via Chi square test

Effects of Chronic Treatment with Fluphenazine and/or Resveratrol on MAO-A and MAO-B Activity

The activities of MAO-A or MAO-B in the cortex and striatum were not modified after chronic treatment with fluphenazine enantate and/or resveratrol (Fig. 5). However, a negative correlation was found between the number of VCMs and MAO-B activity in the striatum [r = −0.42, $p = 0 < 0.05$] (Fig. 6).

Discussion

TD is the most serious side effect caused 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 the disease as well as possibilities for effective treatments are still unknown [10, 46]. In the present study we found that VCMs induced by fluphenazine reduced in rats treated with resveratrol and that there was a negative correlation between the number of VCMs and striatal MAO-B activity.

As previously mentioned, the pathophysiology of TD involves different neurotransmitter and receptor types [10, 17, 37, 47, 48]. However, classical and recent studies continue investigating the dopaminergic system as the central

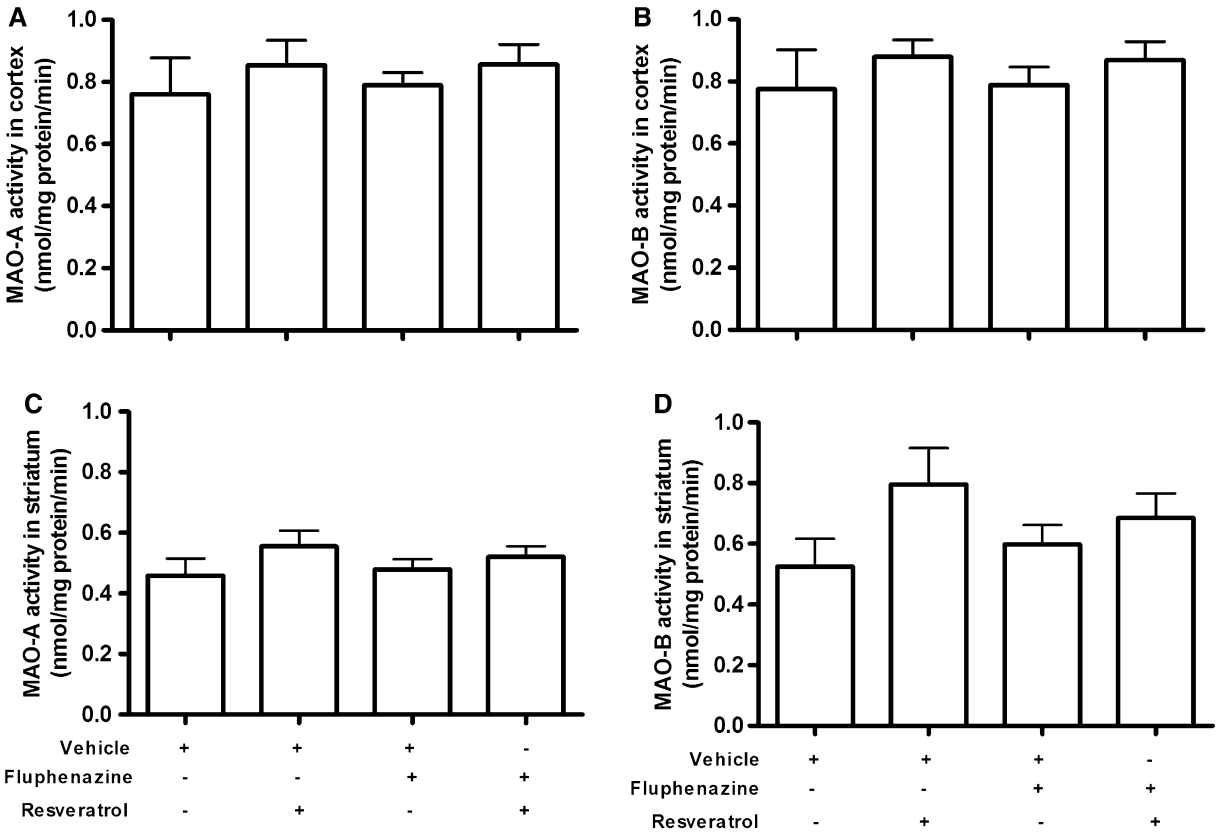

Fig. 5 Effects of chronic treatment (18 weeks) with resveratrol (20 mg/kg, drinking water, every day) and/or fluphenazine enantate [25 mg/kg, intramuscularly (i.m.), every 21 days] on monoamine oxidase (MAO)-A (**a, c**) and MAO-B (**b, d**) activity in the cortex (**a, b**) and striatum (**c, d**) of rats. Data are expressed as mean ± standard error of mean (SEM) [n = 5–9; one-way analysis of variance (ANOVA)]

focus for the development of TD in humans [49] and OD in animals [20, 31, 32], exploring other aspects of this system beyond D_2 supersensitivity [8–10]. Our group has demonstrated that experimental animals with high number of VCMs present a reduction in striatal dopamine uptake after chronic treatment with haloperidol or fluphenazine [31, 32]. Consistent with this, a recent study showed that depletion of monoamines by reserpine causes motor injury in *Caenorhabditis elegans* due to dopaminergic alterations [50]. Of particular importance, in humans, one case report revealed that the improvement in TD symptoms was associated with an increase in dopamine transporter (DAT) levels in striatum [49]. Furthermore, the activation of cannabinoid 1 (CB1) receptor, which indirectly regulate the release of dopamine, decreases VCMs induced by haloperidol in rats [37].

In this scenario, it is important consider the protective action of resveratrol against the dopaminergic neurotoxicity in experimental animals [51, 52]. Our group found that resveratrol in low doses (1 and 5 mg/kg) reduces VCMs induced by reserpine in mice [29] or by fluphenazine in rats in an acute model [30]. These data suggest that resveratrol acts in both models through a common mechanism. However, the mechanism of action 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 [31–33, 35]. Considering this, 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 orally since it is the route through which the population consumes resveratrol in the food. Body weight gain of the animals was used as an indicator 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 the chronic administration of resveratrol. In the literature, we found studies using a wide range of resveratrol doses, varying from 1 to 100 mg/kg [53, 54]. It is important to emphasize that there is no safe dose of resveratrol established for use in humans.

In agreement with our previous data, fluphenazine significantly reduced weight gain in animals [31], and the co-treatment with resveratrol or resveratrol alone did not

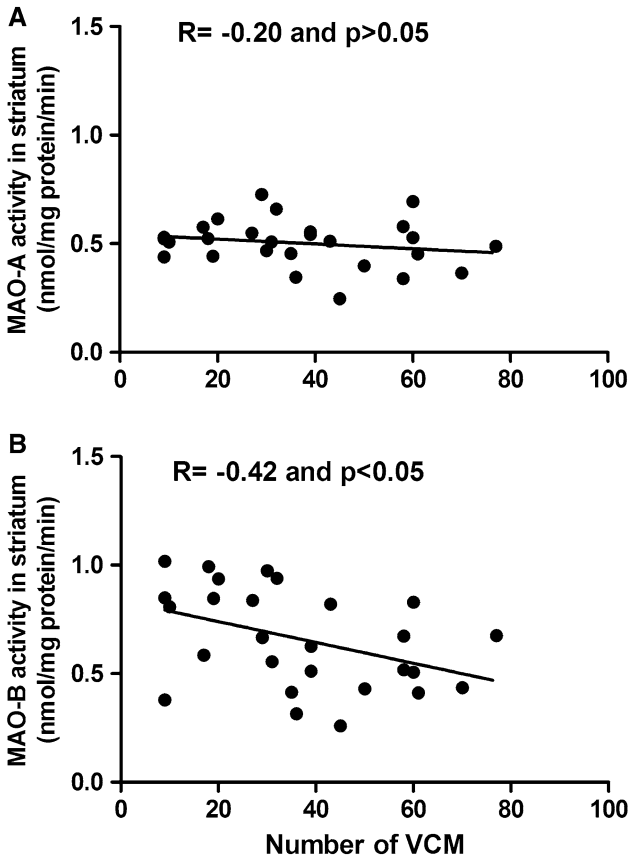

Fig. 6 Correlation between the number of vacuous chewing movements (VCMs) and (**a**) monoamine oxidase (MAO)-A or (**b**) MAO-B activity in the striatum of rats. Correlations (Pearson correlation—r) were considered significant at $p < 0.05$

modify this parameter. Regarding VCMs, the number of VCMs increased after chronic treatment with fluphenazine, and the co-treatment with resveratrol reduced the VCM intensity. As previously demonstrated, fluphenazine administration decreased locomotor and exploratory activity [30, 31]. However, co-treatment with resveratrol did not cause any change in these parameters. Similar responses were observed in acute models using reserpine [29] or fluphenazine [30]. These data suggest that resveratrol exhibits a similar effect in acute and chronic models. Furthermore, the fact that the compound reduced VCMs without altering locomotor and exploratory activity reinforces the idea that it might be acting differentially considering anatomical regions since VCMs have been linked to the ventrolateral neostriatum area, whereas the suppression of locomotion resulting from dopamine depletion to the nucleus accumbens [55, 56].

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 reported the participation of MAO enzyme in these events [10, 20]. Pharmacological effects due the inhibition of MAO-B have been explored for anti-parkinsonian drugs [57] while the inhibition of MAO-A promotes anti-apoptotic and antidepressant effects [18, 19, 58]. Regarding resveratrol, there is evidence that *cis*- and *trans*-resveratrol reduce noradrenaline and 5-hydroxytryptamine uptake and inhibit the activity of both isoforms of MAO [27]. In this study, no difference was found in MAO activity among the groups. The particular absence of effect of resveratrol on MAO activity might be related with the route of administration, which could modify the structure of compound via metabolism; this disrupts its interaction with the enzyme [59] since there is evidence that resveratrol inhibits MAO in vitro [27]. Indeed, resveratrol inhibits MAO in a reversible manner for MAO-B and MAO-A (Busanello et al., unpublished data) which could disrupt the inhibition in ex vivo analysis.

An important effect found in the present study was the relation of VCMs with the MAO-B activity in the striatum. There was a decrease in MAO-B activity in rats that presented the highest number of VCMs, which was observed through negative correlation found among the parameters. In accordance with this, a recent study from our group demonstrated that VCMs induced by reserpine in mice were related to a reduction in MAO activity [20], highlighting the important role of MAO in the development of VCMs in animal models. Acute administration of antipsychotics blocks dopamine receptors blockage and increase dopamine synthesis as well as its metabolism by MAO [10, 16] with consequent reactive species generation [19]. Hence, we suggest that chronically, the MAO enzyme, which is sensible to oxidative stress, had a decrease in its function associated with VCMs maintenance. However, despite antioxidant properties of resveratrol, it did not protect all animals from developing OD, a phenomenon previously observed with other antioxidant substances [29–31, 60].

Conclusion

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

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