

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 270-320 g (\pm 2 months), from our own breeding colony were kept in cages with continuous access to foods and *V. officinalis* or its vehicle (ethanol 1%) in a room with controlled temperature (22 ± 3 °C) and on a 12-h light/dark cycle with lights on at 7:00 am.

Drugs

Haloperidol decanoate (Haldol[®]) was supplied from Janssen Pharmaceutical (São Paulo, Brazil). A standard tincture of *V. officinalis* (10 g of valerian roots per 100 mL of ethanol) was obtained from Bio extracts.

Treatments

The rats were divided into four groups: control group received soy oil (that was the haloperidol vehicle, i.m.) and ethanol 1% in the drink water; *V. officinalis* group received soy oil (i.m.) and *V. officinalis* 1% in the drink water; haloperidol group received haloperidol decanoate (i.m.) and ethanol 1% in the drink water; and haloperidol plus *V. officinalis* group received haloperidol decanoate (i.m.) and *V. officinalis* 1% in the drink water. Haloperidol decanoate or its vehicle were administered intramuscularly (i.m.) every 28 days (38 mg/Kg, i.m.) that is equivalent to 1 mg/kg/day of unconjugated haloperidol. *V. officinalis* was dissolved in the drink water in a proportion of 1%. *V. officinalis* and its vehicle were placed

daily before the beginning of the dark cycle. It was not observed a reduction in liquid intake.

V. officinalis treatment started 15 days before the administration of haloperidol. The treatment with haloperidol was carried out during 12 weeks concomitantly with *V. officinalis*.

Behavioral analysis

Quantification of OD

Behavior measurement of OD was assessed before the treatment with haloperidol or its vehicle (basal evaluation) as previously described. The effect of drugs on behavior was examined every 15 days beginning on the 15th day after the first haloperidol injection (that occurred on same day of the basal behavior) during a period of 12 weeks. To quantify the occurrence of OD, rats were placed individually in cages (20x20x19 cm) and hand operated counters were employed to quantify vacuous chewing movement (VCMs) frequency. VCMs are defined as single mouth openings in the vertical plane not directed towards physical material. If VCMs occurred during a period of grooming they were not taken into account. The behavioral parameters of OD were measured continuously for 6 min after a period of 6 min adaptation. 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.

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). In the present study, we have also verified the prevalence of neuroleptic-induced OD. In our laboratory, control rats present maximally 40 VCMs during a period of 6 min. Thus, in this study, we analyzed the rats that developed neuroleptic-induced OD (+VCM, more than 40 VCMs) separately from those that did not develop neuroleptic-induced OD (-VCMs, less than 40 VCMs), as described by Andreassen et al., 2003, Egan et al., 1994 and Shirakawa and Tamminga, 1994.

Open field test

To analyze the locomotor activity, the animals were placed individually in the center of an open-field arena (40×40×30 cm) with black plywood walls and a white floor divided into 9 equal squares, as previously described (Kerr et al., 2005). The number of line crossings was measured over 2 min and taken as an indicator of locomotor activity.

Elevated plus maze

To evaluate the anxiety state caused by treatment with haloperidol and/or *V. officinalis*, animals were exposed to an elevated plus maze (Chopin et al., 1985; Da Silva et al., 2006). The number of head dippings and the time spent into open or closed arms were recorded over a 2 min session. The percentage of the time spent on open arm and the percentage of the entries into the open arms were calculated, as follows: time spent or number of entries into the open arm/ total time or total number of the entries into closed and open arm X 100, respectively.

Tissue preparations

Rats were killed about 24 hours after the last session of behavioral quantification (on the 28th day after the last administration of haloperidol). The brains were immediately excised and put on ice. The cortex, striatum and region containing the substantia nigra were separated, weighed and homogenized in 10 volumes (w/v) of 10 mM Tris-HCl, pH 7.4. A portion of the striatum was dissected for slices used for the [³H] dopamine uptake assay.

[³H] dopamine uptake

[³H] dopamine uptake was carried out as described by Holz and Coyle (1974) with some modifications. To measure [³H] dopamine uptake, the striatum was cut into 400 µm slices, which were washed with a buffered solution (1) consisting of 127 mM NaCl, 1.2 mM Na₂HPO₄, 5.36 mM KCl, 0.44 mM KH₂PO₄, 0.95 mM MgCl₂, 0.70 mM CaCl₂, 10 mM glucose, and 1 mM Tris-HCl, pH 7.4. Slices (0.2-0.3 mg protein) were further pre-incubated in 96 well-polycarbonate plates for 15 min at 35° C with the buffered solution plus selegiline 1 µM. [³H] dopamine was added to the incubation medium and uptake was carried out for 10 min at 35° C, after which the reaction was stopped by five washes of 30 seconds each with 1 mL of iced-cold solution 1, containing 1 µM selegiline and 100 µM cocaine. Immediately after washing, 0.25 mL of 0.5 M NaOH and 0.2% sodium dodecyl sulfate (SDS) was added to the slices that were digested by 10 min incubation at 60° C. Aliquots of the lysates were taken for protein content measurement by the Lowry et al. (1951) method. For determination of the

intracellular amount of dopamine, liquid scintillation counting was used. Results were expressed as [³H] dopamine uptake per mg of protein.

Oxidative stress parameters

To evaluate the levels of reactive oxygen species (ROS), the homogenates were centrifuged for 10 min at 1,500 x g. Just after the centrifugation, an aliquot of supernatant was used for 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) oxidation. DCFH-DA-oxidation was determined spectrofluorimetrically, using 7 µM of DCFH-DA. Fluorescence was determined at 488 nm for excitation and 520 nm for emission. A standard curve was carried out using increasing concentrations of 2',7'-dichlorofluorescein (DCF) incubated in parallel (Pérez-Severiano et al, 2004). The results were put in percentage in relation to control group.

To assess lipid peroxidation, we quantified thiobarbituric acid (TBA) reactive substances (TBARS). The homogenates were centrifuged for 10 min at 1,500 x g. Just after the centrifugation, an aliquot of 200 µl or of supernatant was incubated for 1 h at 37°C and then used for lipid peroxidation quantification as earlier described (Ohkawa et al., 2004; Rossato et al., 2002).

To verify protein carbonyl, cortical and nigral tissue were homogenized in 10 volumes (w/v) of 10 mM Tris-HCl buffer pH 7.4. The protein carbonyl content was determined by the method described by Yan et al. (1995), with some modifications. Briefly, homogenates were diluted 1:8 in 10 mM Tris-HCl buffer pH 7.4 and 1 ml aliquots were mixed with 0.2 ml of 2,4-dinitrophenylhydrazine (10 mM DNPH) or 0.2 ml HCl (2 M). After incubation at room temperature for 1 h in a dark ambient,

0.5 ml of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing 3% SDS), 2 ml of heptane (99.5%) and 2 ml of ethanol (99.8%) were added sequentially, and mixed with vortex agitation for 40 s and centrifuged for 15 min. Next, the protein isolated from the interface was washed two times with 1ml of ethyl acetate/ethanol 1:1 (v/v) and suspended in 1 ml of denaturing buffer. Each DNPH sample was read at 370 nm against the corresponding HCl sample (blank), and total carbonylation calculated using a molar extinction coefficient of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$ according to Levine et al. (1990).

To verify superoxide dismutase (SOD) activity, cortex, striatum or substantia nigra were adequately diluted to 40 volumes with Tris-HCl 10 mM (pH 7.5) and the assay was performed according to the method of Misra and Firdovich (1972). Briefly, epinephrine rapidly auto oxidizes at pH 10.2 producing adrenochrome, a pink colored product that can be detected at 480nm. The addition of samples (10, 25, 50 μL) containing SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored during 180 seconds at intervals of 30 seconds. The amount of enzyme required to produce 50% inhibition at 25° C was defined as one unit of enzyme activity. The SOD activity was expressed as units/g of protein.

Protein content was measured by method of Lowry et al. (1951) and bovine serum albumin was used as standard.

Statistical Analysis

Data from behavioral parameter were analyzed by two-way ANOVA. 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. Data from TBARS, ROS quantification, SOD activity, carbonyl content and [³H] dopamine uptake were analyzed by one-way ANOVA, followed by Duncan's Post Hoc tests when appropriate. A possible relationship between oxidative stress parameters, VCM, and [³H] dopamine uptake were also determined using linear regression analysis using SPSS 10.1 for Windows. Significance was considered when $p < 0.05$.

RESULTS

Effects of *V. officinalis* on VCM induced by long-term treatment with haloperidol

Haloperidol caused a marked increase on VCM when compared with its vehicle ($F(5, 44)=10.41$ and $p< 0.001$; Figure 1). In fact, a significant interaction between haloperidol and VCM quantifications ($F(30, 264)=2.27$ and $p<0.001$) was observed in this case. Treatment with haloperidol induced an OD prevalence of 40% compared to its vehicle (Qui-square = 4.05; $p<0.05$). The treatment with *V. officinalis* was not able to reduce neither the prevalence nor the intensity of OD in those rats that developed OD. In fact, the co-treatment of haloperidol with *V. officinalis* developed OD in 35.7% of the rats.

Effects of haloperidol and *V. officinalis* on oxidative stress parameters

There was not a significant difference among the groups in DCFH-DA-oxidation levels, TBARS, carbonyl content groups and SOD activity in rats under long-term treatment with haloperidol and *V. officinalis* (Table 1). Furthermore, it was not found any significant relationship between biochemical parameters and brain structures. However, it was possible to observe a significant correlation between SOD activity in the cortex and VCMs ($R^2= - 0.40$ and $p<0.05$; Figure 5A).

Effects of haloperidol and *V. officinalis* on [3H] dopamine uptake

Treatment with haloperidol, in those rats that developed VCM, caused a significant decrease in [3H] dopamine uptake in striatal slices when compared to

control group ($p < 0.05$) (Figure 2). *V. officinalis* co-treatment did not protect against haloperidol-induced [^3H] dopamine uptake reduction in those rats that developed +VCM (Figure 2). In rats co-treated with both drugs and presenting -VCM, the level of [^3H] dopamine uptake was similar to vehicle levels (Figure 2). *V. officinalis* administration alone did not alter [^3H] dopamine uptake in rats treated with vehicle. Interestingly, we found a significant correlation between the DCFH-DA-oxidation in the substantia nigra region and [^3H] dopamine uptake in slices from striatum of rats ($R^2 = -0.29$ and $p < 0.05$; Figure 5B).

Effects of long-term treatment with Valeriana officinalis and haloperidol on locomotor activity in rats

Haloperidol caused a marked and time-dependent decrease on locomotor activity, represented by the number of crossings in the open field test. In fact, a significant interaction between haloperidol and time treatment ($F(3,138) = 12.12$ and $p < 0.001$) was observed. *V. officinalis* administered alone also caused a significant decrease in locomotor activity only after 10 weeks of treatment (Figure 3).

Effects of long-term treatment with V. officinalis and haloperidol on plus maze test in rats

There was a significant effect of the time on head dipping ($F(3,138) = 5.72$ and $p < 0.05$; Figure 4A). Long-term treatment with haloperidol did not cause any effect on head dipping in rats. Similarly, *V. officinalis* alone or with haloperidol also did not cause any effect on this parameter.

Long-term treatment with haloperidol did not cause any effect neither in the percentage of the time spent on open arm nor in the percentage of entries into the open arm (Figure 4B and 4C). *V. officinalis* alone caused a significant increase in the percentage of the time spent on open arm in last observation (Figure 4B). Furthermore, there was a significant difference of *V. officinalis* from other groups in the percentage of the entries into the open arm that started 8 weeks after the first haloperidol administration (Figure 4C). The co-treatment with *V. officinalis* and haloperidol did not cause neither effect in the percentage of the time spent into open (Figure 4B) nor in the percentage of entries into the open arm (Figure 4C). Also, a significant effect of the time was observed in the percentage of time spent into the open arm ($F(3, 138) = 3.99; p < 0.05$) and in the number of entries into the open arm ($F(3, 138) = 3.35; p < 0.05$).

DISCUSSION

TD is a serious side effect caused by long-term treatment with neuroleptic drugs. Particularly, it is problematic due to its high prevalence and the lack of effective treatment. Our current study show that *V. officinalis* was not effective in reduces OD prevalence or intensity in rats under chronic treatment with haloperidol. *V. officinalis* showed a significant effect to maintain rats on the open arm of the elevated plus maze. The chronic treatment with *V. officinalis* and/or haloperidol did not cause any effect on oxidative stress parameters. Furthermore, the reduction in dopamine uptake in striatum seems to have an important role in the development of OD in rats, an effect not reversed by chronic treatment with *V. officinalis*.

It has been demonstrated that long-term treatment with neuroleptic drugs is capable of producing OD in rats and TD in humans. However the mechanisms that can be involved are not clear. In the present study, we found that long-term treatment with haloperidol caused a prevalence of OD in 40% of treated rats. Accordingly, a previous study showed that chronic treatment with haloperidol develops significant OD 45-55% in rats with 6 months of treatment and approximately 65-75% after 12 months of treatment (Kaneda et al, 1992).

Although the etiology of TD is unclear, reduced GABA is thought to be important in this syndrome. In fact, it has been described a decrease in the GAD activity and in the levels of GABA in brain regions of monkeys with dyskinetic symptoms induced by neuroleptics (Gunne et al, 1984). Accordingly, recent data from literature have demonstrated that GABA-mimetic drugs can provide a

protective role on OD induced by reserpine and neuroleptics in rats (Kaneda et al, 1992; Gao et al, 1994; Raghavendra et al, 2001; Araujo et al, 2005). Furthermore, there are some human studies where GABA agonists were shown to improve TD (Tamminga et al, 1979; 1983; Morselli et al, 1985; reviewed in Tamminga et al, 1989; Soares et al, 2004). *V. officinalis* is among the most widely used herbal medicines used for centuries as a calming and sleep promoting herb (Fugh-Berman and Cott, 1999; McCabe, 2002; Lustberg and Reynolds, 2000; Morazzoni and Bombardell, 1995). In this way, *V. officinalis* could to be efficacious against TD since its mechanism of action seems to be related with the potentiation of GABAergic transmission via agonist effect at benzodiazepine site of GABA_A receptor, induction of GABA release and inhibition of GABA reuptake (Santos et al, 1994; Ortiz et al, 1999; Mennini et al, 1993). However, *V. officinalis* treatment was not able to alter the prevalence or the intensity of haloperidol-induced OD. Thus, *V. officinalis* seems not promote any beneficial effect on OD, at least in a well-described animal model.

V. officinalis is clinically used to relieve anxiety and improve symptoms of insomnia (McCabe, 2002; Lustberg and Reynolds, 2000; Morazzoni and Bombardell, 1995; Kennedy et al, 2006). Thus, we investigated the effects of *V. officinalis* in the locomotor activity and anxiety to evaluate if the treatment was capable of producing pharmacological effects. Showing effectiveness of our treatment, *V. officinalis* was capable of producing hypolocomotion and anxiolytic effect in the treated rats when assessed in open field and plus maze tests 10 weeks after the beginning of the treatment. In accordance with our findings, it has

been demonstrated that acute and chronic treatment with *Valeriana* possesses similar effects on anxiety parameters (Della Loggia et al, 1981; Sakamoto et al, 1992; Oliva et al, 2004). However, *V. officinalis* did not reduce OD induced by haloperidol in rats, even after 10 weeks of treatment.

A recent hypothesis postulated that free radicals could have an important role in the development of TD (Lohr et al, 1990; 2003). It is known that the initial action of neuroleptic treatment is to cause a secondary increase in dopamine turnover, which, in turn, increase the metabolism of dopamine by monoamino oxidase (MAO) and, hydrogen peroxide (H_2O_2) can be formed in this way (Andreassen and Jorgensen, 2000; Lohr, 1991; Lohr et al, 2003). Moreover, the own autoxidation of dopamine can form dopamine quinone that acts as reactive specie (Lohr et al, 2003). There are some studies showing that patients with TD had an increase in oxidative stress parameters in plasma and CSF (Brown et al., 1998; Lohr et al, 1990; Pall et al, 1987; Tsai et al, 1998). In rats, acute OD has been related to an increase in oxidative stress parameters (Abílio et al, 2004; Andreassen et al, 2003; Naidu et al, 2003; Burger et al, 2005a,b; Faria et al, 2005) and treatment with antioxidant substances seems to be efficacious to reduce OD (Singh et al, 2003; Naidu et al, 2003; Burger et al, 2003; 2004; 2005a). However, oxidative stress seems to be important in beginning of the events that culminate in OD. In fact, it was detected increase in OD and oxidative stress in several brain regions one month after haloperidol treatment in rats (Naidu et al, 2003; Burger et al., 2005a). On the other hand, we have detected increased OD, but not oxidative stress in the same brain regions 7 months after haloperidol treatment (Fachinetto

et al., 2005). Here, we did not also find any alteration in oxidative stress parameters evaluated after 3 months under neuroleptic treatment. Nevertheless, a significant correlation between SOD activity in cortex and VCM quantity was observed. The negative correlation shows that the rats presenting high VCM had the lesser activity of SOD in cortex. Interestingly, some studies have reported alterations in cortical parameters of oxidative stress in rats receiving acute treatment with neuroleptic drugs (Burger et al, 2005a, Balijepalli, 2001).

The clinical use of medicinal herbs has been demonstrated to be efficacious to treat several diseases. However, such treatment is also subject to develop important side-effects (Kumar, 2006). It was previous reported that the treatment with *V. officinalis* during 7 days caused oxidative stress in liver of mice (Al-Majed et al, 2006). However, an important finding of our study was that the chronic treatment with *V. officinalis* did not cause any alteration on oxidative stress parameters neither in the central nervous system (CNS) nor in liver and kidney (F.A.A Soares, unpublished data). More studies must be carried out to elucidate the toxic potential of *V. officinalis* treatment.

We also demonstrated that there was a significant reduction in dopamine uptake in the animals presenting OD in relation to the control group and group that did not develop OD. *V. officinalis* could not neither prevent the reduction in dopamine uptake nor OD in those rats. Several factors might explain the reduction of dopamine uptake in the striatum of rats presenting OD, including neurodegeneration of cells that uptake dopamine and altered dopamine transport function. It has been shown that some neuroleptics, including haloperidol, can

directly interact with and inhibit the dopamine transporter (Lee et al., 1997). In addition to this putative mechanism, literature data have shown that oxidative stress can decrease the activity of dopamine transporters (Huang et al., 2003; Hashimoto et al, 2004). Accordingly, we find a negative correlation between the DCFH-DA-oxidation in the substantia nigra region and [³H] dopamine uptake in slices from striatum of rats. In addition to dopamine transport function mechanism, it was reported that the development of OD in rats is associated with histopathological alterations in the substantia nigra suggesting that nigral degeneration may contribute to the development of persistent VCM in rats (Andreassen et al, 2003). Since the projection of the nigral neuron is responsible to release and reuptake of dopamine in the striatum, the neurodegeneration in nigral cells could also explain the reduction in dopamine uptake. However, further studies must be carried out to elucidate the exact mechanisms through haloperidol-treatment reduces dopamine uptake.

Taken together, our data suggest that the oxidative stress seems not to have an important role in maintenance of OD. Moreover, a mechanism involving the reduction of dopamine transport related with the maintenance of chronic OD in rats. *V. officinalis* was not able to prevent OD as well as reverse dopamine uptake levels in those rats that developed OD rats, thus, its chronic treatment seems not produce any oxidative damage to CNS. Therefore, *V. officinalis* seems to be devoid of effect to prevent or treat OD.

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Legends for figures:

Figure 1: Effects of *V. officinalis* on haloperidol-induced orofacial dyskinesia. Number of vacuous chewing movements (VCM) for 6 min during long-term treatment. Values are presented as means \pm S.E.M. (Control, n=12; *V. officinalis*, n = 14; haloperidol (-VCM), n = 6, haloperidol (+VCM), n = 4; haloperidol + *V. officinalis* (-VCM), n = 9; haloperidol + *V. officinalis* (+VCM), n = 5).

Figure 2: Effects of long-term treatment with haloperidol and *V. officinalis* on [3 H] dopamine uptake (CPMA/mg protein) in slices from striatum of rats. Data were analyzed by one-way ANOVA followed by Duncan's multiple range tests.

Figure 3: Effects of *V. officinalis* on open field test in rats. Number of crossings in 2 min. Values of number of crossings are presented as means \pm S.E.M. (Control, n=12; *V. officinalis*, n = 14; haloperidol , n = 10; haloperidol + *V. officinalis*, n = 14. Symbols represent significant differences among the groups in the same period of observation.

Figure 4: Effects of *V. officinalis* on plus maze test in rats. A) Number of head dippings for 2 min, B) percentage of the time spent on the open arms for 2 min, C) percentage of entries into the open arms. Values are presented as means \pm S.E.M. (Control, n=12; *V. officinalis*, n = 14; haloperidol , n = 10; haloperidol + *V. officinalis*, n = 14. (one way ANOVA followed by Duncan's multiple range tests).

*represents significant differences among the groups in the same period of observation

Figure 5: Linear regression analysis between SOD activity in cortex and VCM (A) and between DCFH-DA oxidation in substantia nigra and [3H] dopamine uptake (B) in striatum of rats following chronic treatment with haloperidol and *V. officinalis*.

Table 1: Effects of haloperidol and *V. officinalis* treatments on oxidative stress parameters (Mean \pm S.E.M) (Val: *V. officinalis* treatment Halo: Haloperidol treatment).

Brain regions	Lipid peroxidation	ROS levels	SOD activity	Protein carbonyl
	(nmol of MDA/g tissue)	(% of control)	(U/mg protein)	(nmol carbonyl/mg protein)
Cortex				
Control	95.5±5.7	112.0±16.5	377.5±39.6	26.8±3.0
Val	93.9±6.4	92.8±6.1	434.1±29.9	25.6±2.7
Halo -VCM	89.0±9.9	84.1±9.8	348.7±65.4	30.2±2.3
Halo +VCM	85.5±6.1	87.0±13.4	426.6±97.7	28.0±5.2
Val+Halo -VCM	104.5±8.2	88.0±5.5	382.0±60.8	33.0±3.6
Val+Halo +VCM	99.2±15.9	107.8±11.6	425.1±109.4	34.7±10.8
Striatum				
Control	62.2±6.7	107.5±16.7	722.7±77.1	-
Val	64.9±6.1	95.1±8.4	781.7±48.2	-
Halo -VCM	49.6±2.4	131.1±22.3	721.3±92.4	-
Halo +VCM	50.4±7.2	83.0±21.8	614.8±93.9	-
Val+Halo -VCM	55.9±7.2	103.7±8.9	653.8±92.2	-
Val+Halo +VCM	49.4±4.9	143.8±27.5	787.3±124.0	-
Substantia nigra				
Control	88.9±10.5	75.1±9.0	388.8±56.4	29.7±3.4
Val	76.6±3.9	87.1±4.2	459.3±52.1	30.7±3.1
Halo -VCM	65.9±2.9	87.5±9.6	400.3±46.5	34.8±9.6
Halo +VCM	66.8±9.3	97.5±9.9	533.0±39.3	24.4±0.6
Val+Halo -VCM	82.2±9.8	89.1±4.2	433.8±59.4	30.5±3.3
Val+Halo +VCM	80.5±10.0	100.6±11.5	481.1±56.3	28.1±3.1

Figure 1:

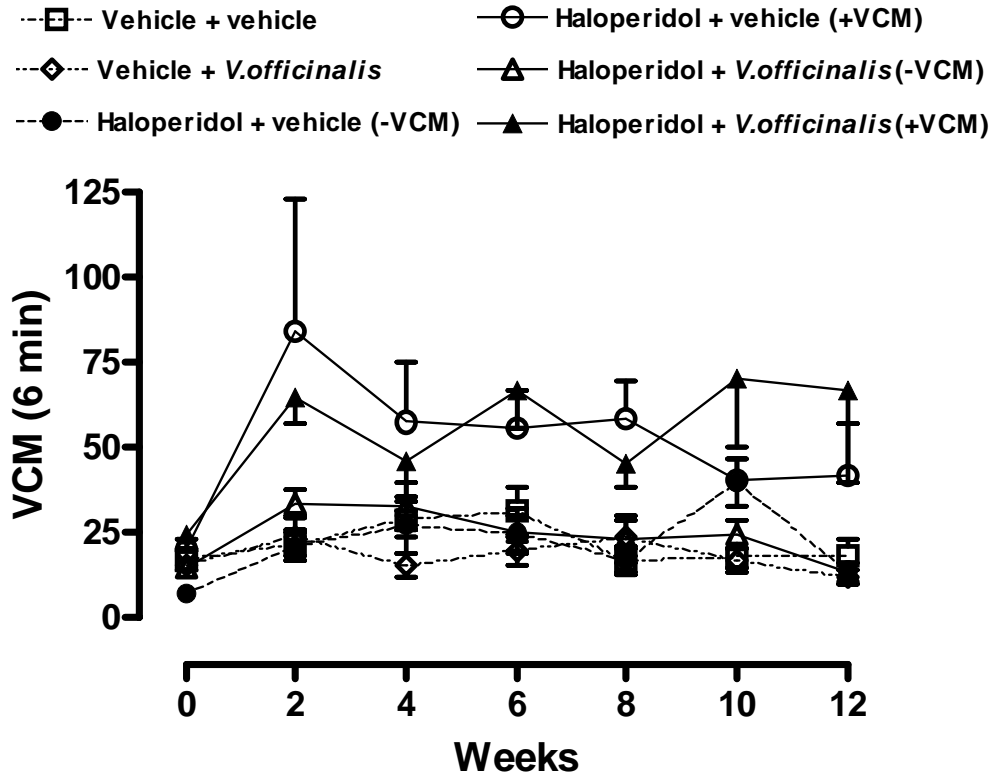


Figure 2:

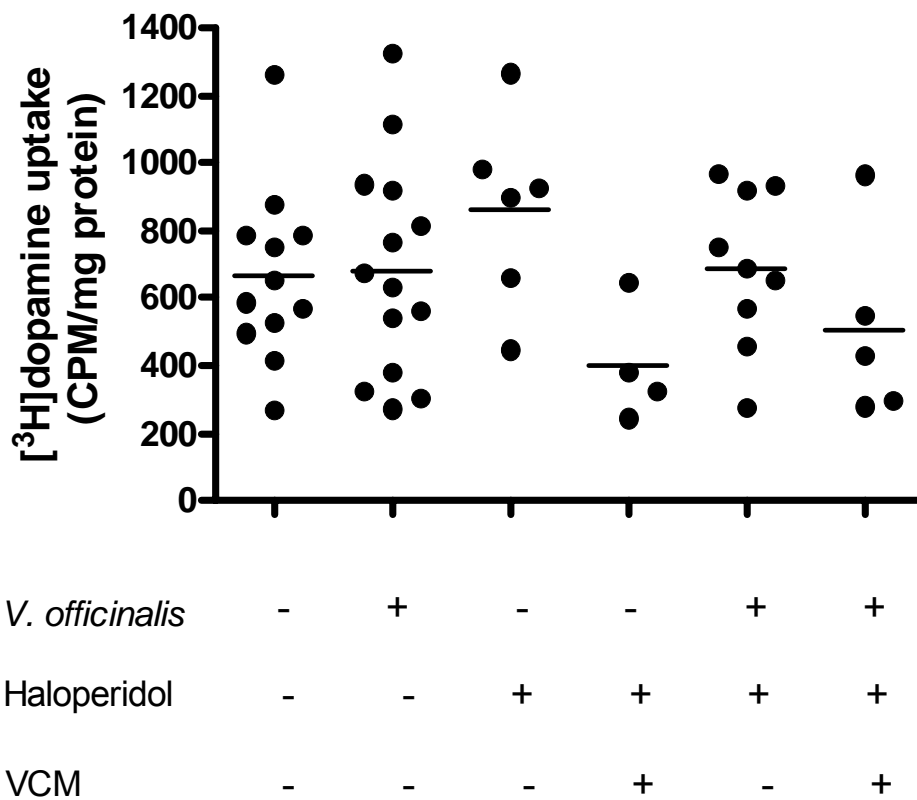


Figure 3:

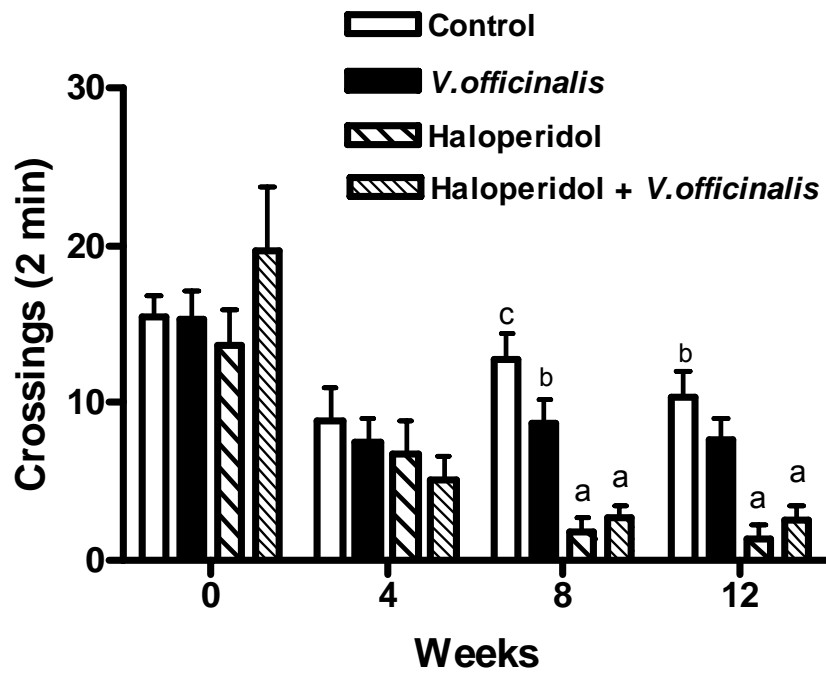


Figure 4:

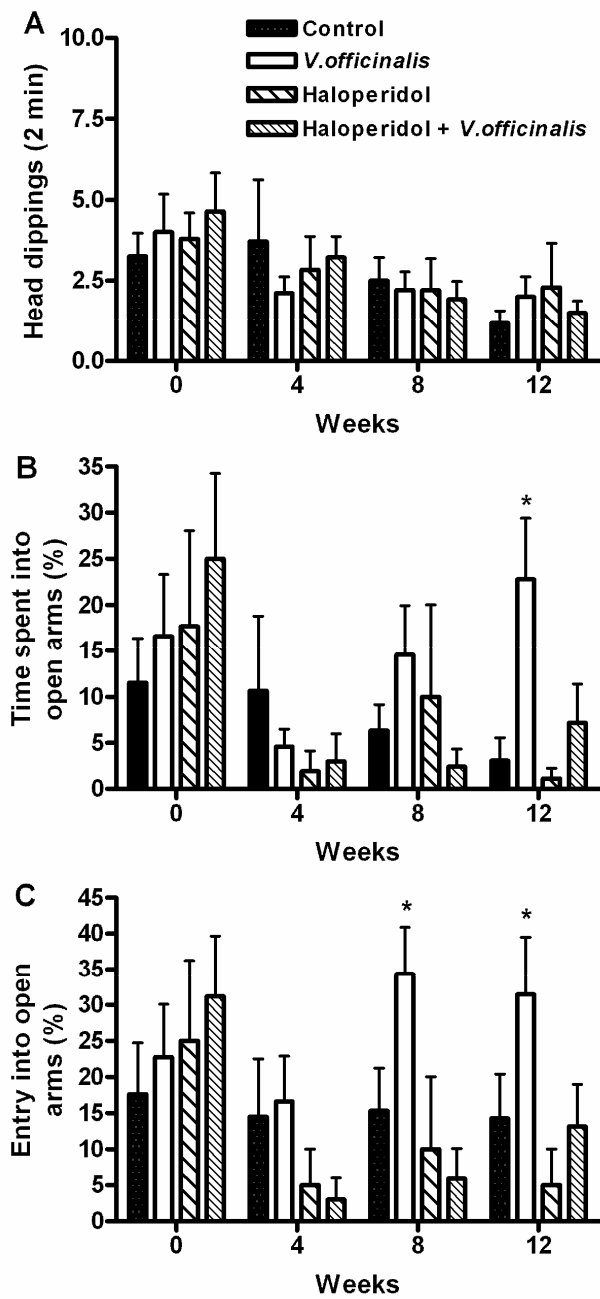
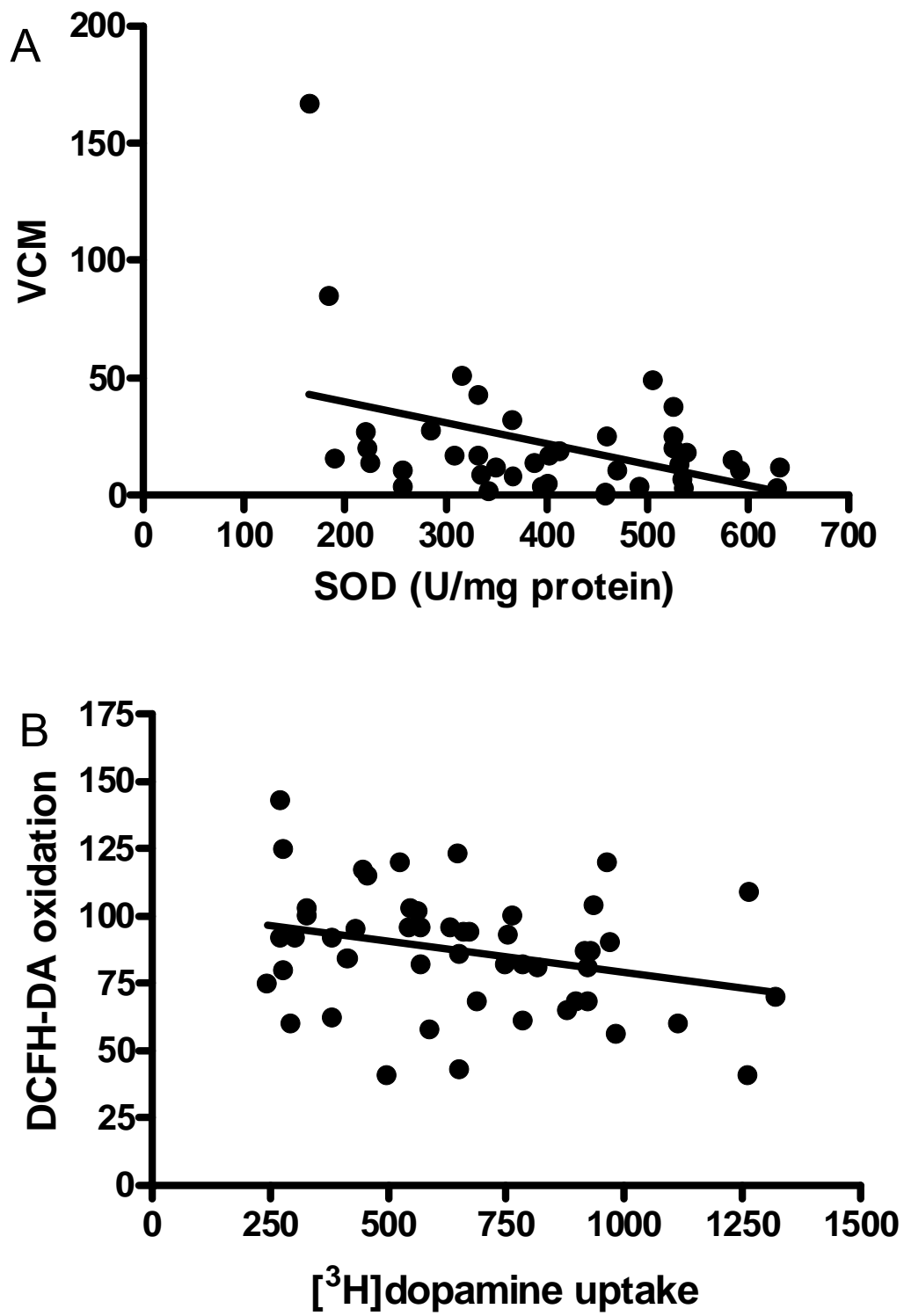


Figure 5:



CONCLUSÕES PARCIAIS 2

A partir do segundo trabalho podemos concluir que:

- O tratamento com *V. officinalis* não foi capaz de prevenir o desenvolvimento da DO.
- O uso crônico de *V. officinalis* não produziu nenhum efeito tóxico observável.
- A manutenção da DO em ratos parece envolver a redução no transporte de dopamina.

4. DISCUSSÃO

A DT consiste no principal problema associado ao uso prolongado de neurolépticos. Particularmente, a DT é problemática devido a sua alta prevalência e falta de um tratamento que seja efetivo. Apesar da grande quantidade de trabalhos propondo mecanismos para o desenvolvimento da DT, sua patofisiologia ainda é um campo obscuro para os pesquisadores.

O presente estudo teve como primeiro objetivo avaliar o efeito de dietas hiper- (HL) e normolípicas (NL) sobre a DO induzida por haloperidol em ratos (artigo 1). Os resultados deste estudo indicam que a dieta HL causou um aumento na intensidade da DO em ratos. Contudo, este efeito foi transitório desaparecendo um mês depois. Além disso, observamos que a concomitante ingestão de dieta HL com a administração de haloperidol resultou em aumento de DO em ratos. Este efeito foi também transitório ficando menos evidente com o passar do tempo. No caso, dos MMV, a redução na intensidade deste parâmetro coincidiu com um aumento na DO dos ratos mantidos com dieta NL. No entanto, para o caso do tremor facial (TF) a redução na intensidade foi conseqüência de uma mudança mais complexa na DO de ambos os grupos. Um fator que pode ter contribuído para a modificação da intensidade de TF é a redução na ingestão após prolongada administração de haloperidol. De fato, os efeitos do haloperidol são complicados devido à relativa perda de massa corporal, sendo que este efeito foi mais exagerado no grupo que recebia haloperidol e dieta HL. Este efeito na perda de massa corporal poderia, em parte, explicar algumas das variações ocorridas. Esta hipótese, embora alternativa, está de acordo com dados da literatura indicando que a restrição alimentar reduz a produção de estresse oxidativo em mamíferos (Armeni e cols., 2003) e pode liberar fatores neurotróficos (Mattson, 2000). Estes resultados indicam que as duas medidas comportamentais não refletem necessariamente os mesmos efeitos patofisiológicos dos neurolépticos sendo que estes podem ser modulados independentemente por fatores exógenos, incluindo idade e restrição alimentar. De acordo com isso, em um estudo prévio, observamos que o ebsellem (Burger e cols., 2003), um agente antioxidante, teve efeito diferente sobre os parâmetros comportamentais em ratos tratados com reserpina.

Dados da literatura indicam que a ação inicial do tratamento com neurolépticos é causar um aumento secundário na renovação de dopamina, o que, pode levar a um aumento no metabolismo da dopamina pela MAO e peróxido de hidrogênio pode ser formado desta maneira. Além disso, a própria autoxidação da dopamina pode formar dopamina quinona que age como espécie reativa (Lohr e cols., 1991; Andreassen e Jorgensen, 2000; Lohr e cols., 2003). De fato, existem alguns estudos mostrando que pacientes com DT possuem um aumento nos parâmetros de estresse oxidativo no plasma e fluido cérebro espinhal (Pall e cols., 1987; Lohr e cols., 1990; Tsai e cols., 1998). Além disso, tem sido demonstrado que a exposição ao haloperidol causa um aumento no estresse oxidativo cerebral (Tse e cols., 1976; Clow e cols., 1980; Slivka e Cohen, 1985; Lohr, 1991; Casey, 1995; Andreasen e Jorgensen, 2000; Abílio e cols., 2004) que pode estar ligado de forma causal a um aumento da DO após tratamento com neurolépticos. Os resultados deste trabalho indicam que o consumo prolongado de dieta HL causou um aumento de estresse oxidativo em córtex e cerebelo, como indicado por um significativo efeito da dieta independente do tratamento com haloperidol. De particular importância para a DO, o haloperidol causou um aumento na produção de TBARS no grupo da dieta HL especificamente nas regiões do cérebro que são descritas como sendo envolvidas na gênese da DT, o estriado e a região contendo a *substantia nigra* (Tsai e Ikonomidou, 1995; Andreassen e Jorgensen, 2000; Lohr e cols., 2003). Contudo, o aumento na produção de TBARS nestas regiões não pode exclusivamente levar a um aumento na DO, devido à falta de diferenças significativas nos parâmetros de DO entre os grupos das duas dietas e tratados com haloperidol, no final do período de observação.

Em conjunto, os resultados deste estudo indicam que a ingestão de dieta HL por um período prolongado pode ter alguns efeitos comportamentais transitórios em ratos. Além disso, demonstramos pela primeira vez que a ingestão simultânea de dieta HL e administração crônica de haloperidol causam exacerbação transitória na DO em ratos, contudo o modelo animal pode não refletir os mesmos efeitos em humanos. Apesar de os dados da literatura indicarem que a DO induzida por neurolépticos está associada com estresse oxidativo, fomos incapazes de estabelecer alguma correlação, porque o haloperidol aumentou o estresse oxidativo apenas em cérebro de ratos mantidos com dieta HL, já a intensidade da DO foi similar entre os grupos das dietas HL e NL. Uma possível explicação

para estes resultados reside numa antecipação do estresse oxidativo em ratos ingerindo dieta HL e tratados com haloperidol que é anterior ao desenvolvimento de DO em ratos. Isto está de acordo com Andreassen e cols. (1998) e Calvent e cols. (2002) onde a administração de ácido propiônico potencializou a DO de ratos. O desaparecimento de efeitos entre os grupos de dieta NL e HL pode ser consequência de uma complexa interação com fatores que afetam a DO em ratos, particularmente os de maior idade (Kane e Smith, 1982; Woerner e cols., 1991; Yassa e Jeste, 1992) ou outros mecanismos compensatórios como plasticidade da neurotransmissão que poderiam levar a consequências neuronais de estresse oxidativo podendo ser influenciado pela idade.

O segundo objetivo deste estudo foi avaliar os efeitos da tintura de *V. officinalis* sobre a DO induzida por haloperidol em ratos (artigo 2). Este estudo demonstrou que a *V. officinalis* não foi efetiva em reduzir a DO em ratos tratados cronicamente com haloperidol. Contudo, o tratamento crônico com *V. officinalis* não causou nenhum efeito sobre parâmetros de estresse oxidativo em ratos. Além disso, a redução na captação de dopamina em estriado parece ter um importante papel na DO em ratos e o tratamento crônico com *V. officinalis* não foi capaz de revertê-la.

Tem sido demonstrado que o tratamento com drogas neurolépticas é capaz de produzir DO em ratos e DT em humanos. Neste estudo, o tratamento com haloperidol causou uma prevalência de DO de 40%. De acordo com isso, um estudo prévio demonstrou que o tratamento crônico com haloperidol desenvolve DO em cerca de 40-55% em ratos tratados durante um período de seis meses, sendo que este índice aumenta para cerca de 65-75% após 12 meses de tratamento (Kaneda e cols., 1992).

Embora a etiologia da DT não seja clara, a redução no GABA parece ser importante. De fato, tem sido descrito uma redução na atividade da enzima glutamato descarboxilase e nos níveis de GABA em regiões do cérebro de macacos com sintomas discinéticos induzidos por haloperidol (Gunne e cols., 1984). De acordo com isto, recentes dados da literatura têm demonstrado que drogas GABA-miméticas podem ter um papel protetor na discinesia orofacial induzida por reserpina e neurolépticos em ratos (Kaneda e cols., 1992; Gao e cols., 1994; Raghavendra e cols., 2001; Araujo e cols., 2005). Além disso, existem alguns estudos em humanos onde agonistas GABAérgicos melhoraram os sintomas da DT (Tamminga e cols., 1979; 1983; revisado por Tamminga e cols., 1989; Soares e cols.,

2004). *V. officinalis* está entre as ervas medicinais mais utilizadas como calmante e indutor de sono (Morazzoni e Bombardell, 1995; Fugh-Berman e Cott, 1999; Lustberg e Reynolds, 2000; McCabe, 2002). Deste modo, *V. officinalis* poderia ser benéfica impedindo o desenvolvimento da DT desde que seu mecanismo de ação parece ser relacionado a uma potencialização na neurotransmissão GABAérgica via efeito agonista em receptor GABA_A, indução da liberação e inibição da recaptação de GABA (Mennini e cols., 1993; Santos e cols., 1994; Ortiz e cols., 1999). No entanto, o tratamento com *V. officinalis* não foi capaz de alterar a prevalência e nem intensidade da DO induzida por haloperidol. Desta forma, *V. officinalis* parece não ter nenhum efeito benéfico sobre DO, pelo menos no modelo animal descrito.

A *V. officinalis* é utilizada para o alívio de ansiedade e melhora dos sintomas de insônia. Então, investigamos os efeitos da *V. officinalis* na atividade locomotora e ansiedade em ratos com o objetivo de avaliar se o tratamento estava produzindo efeito farmacológico (Morazzoni e Bombardell, 1995; Lustberg e Reynolds, 2000; McCabe, 2002; Kennedy e cols., 2006). De fato, a *V. officinalis*, na forma como foi administrada apresentou redução na atividade locomotora avaliada pela atividade dos animais no campo aberto e demonstrou efeito ansiolítico observado pelo maior tempo de permanência dos animais no braço aberto do labirinto em cruz.

Com relação ao estresse oxidativo, este parece ser mais importante no início dos eventos que culminam em DO. Em um estudo anterior detectamos aumento de DO e estresse oxidativo em várias regiões do cérebro de ratos 4 semanas após tratamento com haloperidol (Burger e cols., 2005). Por outro lado, observamos aumento de DO, mas não de estresse oxidativo nas mesmas regiões do cérebro de ratos após 7 meses de tratamento com haloperidol (**artigo 1**). No presente estudo, não encontramos alterações nos parâmetros de estresse oxidativo avaliados 12 semanas após tratamento com neurolépticos. Entretanto, foi observada uma significativa correlação entre a atividade da SOD e quantidade de MMV. A correlação negativa entre esses dois parâmetros indica que ratos com maior quantidade de MMV apresentaram menor atividade da SOD. De fato, alguns estudos relatam alterações em parâmetros corticais de estresse oxidativo em ratos recebendo tratamento agudo com fármacos neurolépticos (Burger e cols., 2005a; Balijepalli, 2001).

Demonstramos também que houve uma significativa redução na captação de dopamina nos animais apresentando DO em relação ao grupo que não desenvolveu DO. *V. officinalis* não preveniu nem a redução na captação de dopamina nem a DO em ratos. A redução na captação da dopamina relacionada com o desenvolvimento de DO não tinha ainda sido demonstrada na literatura, consistindo numa nova hipótese para o desenvolvimento da DT. Em um recente trabalho (submetido para publicação), demonstramos que existe uma redução na captação de dopamina em animais tratados cronicamente com flufenazina e que desenvolveram DO, evidenciando que esta não é uma ação particular do haloperidol. Além disso, neste mesmo estudo, o disseleneto de difenila, um organocalcogênio que apresentou eficácia na reversão da DO em tratamento agudo (Burger e cols., 2004; 2006), reduziu a prevalência da DO, sendo este efeito relacionado ao reestabelecimento da captação de dopamina em modelo crônico de DO. Vários fatores podem explicar a redução na captação de dopamina em estriado de ratos apresentando DO, incluindo neurodegeneração celular e alteração na função do transporte de dopamina. Foi demonstrado que alguns neurolépticos, incluindo o haloperidol, podem interagir diretamente reduzindo a atividade do transportador de dopamina (Lee e cols., 1997). Contribuindo para este mecanismo proposto, dados da literatura têm demonstrado que o estresse oxidativo pode reduzir a atividade dos transportadores de dopamina (Huang e cols., 2003; Hashimoto e cols., 2004). De acordo com isso, encontramos uma correlação negativa entre a oxidação do diacetato de diclorofluoresceína (DCFH-DA) na *substantia nigra* e captação de [³H] dopamina em fatias de estriado de ratos. Além disso, foi relatado que o desenvolvimento de DO em ratos está associado com alterações histopatológicas na *substantia nigra*, sugerindo que a degeneração nigral pode contribuir para o desenvolvimento de MMV persistentes em ratos (Andreassen e cols., 2003). Desde que a projeção dos neurônios nigrais é responsável pela liberação e recaptação de dopamina no estriado, a neurodegeneração das células nigrais pode também explicar a redução na captação de dopamina. Contudo estudos posteriores devem ser efetuados para elucidar os exatos mecanismos através dos quais o tratamento com haloperidol reduz a captação de dopamina.

O conhecimento da toxicologia das ervas medicinais é de importância fundamental para o uso seguro destes medicamentos pela população. Foi recentemente relatado que o

tratamento com *V. officinalis* durante 7 dias causou estresse oxidativo em fígado de camundongos (Al-Majed e cols., 2006). No entanto, em nosso estudo, o tratamento crônico com *V. officinalis* durante 14 semanas não causou alterações sobre os parâmetros de estresse oxidativo no sistema nervoso central de ratos, em uma dose que teve efeito farmacológico, embora não apresente efeito sobre a DO induzida por neurolépticos. A toxicidade do tratamento crônico com *V. officinalis* também foi investigada em outros órgãos como fígado e rins destes animais e, nenhum efeito sobre parâmetros de estresse oxidativo foi encontrado (F.A.A. Soares, dados não publicados).

Em conjunto, nossos dados sugerem que o estresse oxidativo pode ter um importante papel no desenvolvimento de DO e um mecanismo envolvendo a redução no transporte de dopamina pode estar relacionado com a manutenção da DO crônica em ratos. *V. officinalis* não foi capaz de prevenir o desenvolvimento da DO bem como reverter os níveis de captação de dopamina naqueles ratos que desenvolveram DO. Contudo, o tratamento crônico com *V. officinalis* parece não produzir estresse oxidativo ao SNC.

5. CONCLUSÕES FINAIS

De acordo com os resultados apresentados nesta dissertação podemos concluir que:

- fatores pró-oxidantes, representados pela dieta hiperlipídica, podem exacerbar a DO induzida por haloperidol em ratos, no entanto, a *V. officinalis*, embora tendo propriedades antioxidantes, não foi eficaz na reversão da DO;
- o estresse oxidativo parece estar envolvido no desenvolvimento da DO, no entanto, após tratamento prolongado com haloperidol não observamos diferenças com relação ao controle;
- a redução da captação de dopamina parece estar envolvida no desenvolvimento da DO e sua manutenção aos níveis normais parece ser o mecanismo que impede seu desenvolvimento.

6. PERSPECTIVAS

Com base nos resultados obtidos no presente trabalho, faz-se necessário:

- investigar o efeito de outras drogas neurolépticas na captação de dopamina, tendo em vista que a redução da captação de dopamina parece estar diretamente relacionada ao desenvolvimento da DO;
- estudar a partir de que período de tratamento com neurolépticos começa a acontecer essa redução na captação de dopamina e como estão os parâmetros de estresse oxidativo no cérebro de ratos neste mesmo período de tratamento;
- avaliar a atividade do transportador de dopamina quando expostos a diferentes concentrações de neurolépticos, *in vitro*, bem como a possível reversão do efeito por agentes como o disseleneto de difenila;
- investigar possíveis alterações na expressão protéica dos transportadores de dopamina;
- determinar o nível de viabilidade celular em estriado de animais após tratamento prolongado com neurolépticos.

DEMAIS TRABALHADOS REALIZADOS DURANTE O PERÍODO DO MESTRADO:

BRITO, V.B., FOLMER, V., PUNTEL, G. O., FACHINETTO, R., SOARES, J.C.M., ZENI, G., NOGUEIRA, C.W., ROCHA, J.B.T. Diphenyl Diselenide and 2,3-Dimercaptopropanol increase the PTZ-induced chemical seizure and mortality in mice. *Brain Res Bull.*, 68, 414-418, 2006.

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BURGER, M.E., FACHINETTO, ALVES, A., CALEGARI, L., ZENI, G., ROCHA, J.B.T. Acute reserpine and subchronic haloperidol treatments change synaptosomal brain glutamate uptake and elicit orofacial dyskinesia in rats. *Brain Res.*, 1031, 202-210, 2005.

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FACHINETTO, R., VILLARINHO, J.G., WAGNER, C., PEREIRA, R.P., PUNTEL, R.L., PAIXÃO, M.W., BRAGA, A. L., CALIXTO, J.B., ROCHA, J.B.T., FERREIRA, J. Diphenyl diselenide decreases the prevalence of orofacial dyskinesia induced by fluphenazine in rats. *Neuropharmacology* (Submetido).

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