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Protective effects of *Polygala paniculata* extract against methylmercury-induced neurotoxicity in mice

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Abstract

We have examined the possible protective effects of *Polygala paniculata* extract against methylmercury (MeHg)-induced neurotoxicity in adult mice. MeHg was diluted in drinking water (40 mg L^{-1}, freely available) and the hydroalcoholic *Polygala* extract was diluted in a 150 mM NaCl solution and administered by gavage (100 mg kg^{-1} b.w., twice a day). After a two-week treatment, MeHg exposure significantly inhibited glutathione peroxidase and increased glutathione reductase activity, while the levels of thiobarbituric acid reactive substances were increased in the cerebral cortex and cerebellum. These alterations were prevented by administration of *Polygala* extract, except for glutathione reductase activity, which remained elevated in the cerebral cortex. Behavioural interference in the MeHg-exposed animals was evident through a marked deficit in the motor performance in the rotarod task, which was completely recovered to control levels by *Polygala* extract co-administration. This study has shown, for the first time, the in-vivo protective effects of *Polygala* extract against MeHg-induced neurotoxicity. In addition, our findings encourage studies concerning the beneficial effects of *P. paniculata* on neurological conditions related to excitotoxicity and oxidative stress.

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Introduction

Methylmercury (MeHg) is one of the most dangerous environmental pollutants. It is a highly neurotoxic compound leading to neurological and developmental deficits in animals and man (Clarkson et al 2003). Although MeHg-induced neurotoxicity is an extensively reported phenomenon, the molecular mechanisms underlying its toxicity are not fully understood. The major mechanisms involved in MeHg neurotoxicity currently being explored are the impairment of intracellular calcium homeostasis (Sirois & Atchison 2000), oxidative stress (Ou et al 1999) and the alteration of glutamate homeostasis (Aschner et al 2000; Farina et al 2003a; Manfroi et al 2004). The last is, at least in part, the main mechanism responsible for the excitotoxic effects observed in MeHg poisoning.

Notwithstanding the massive efforts in the search for new drugs that counteract mercurial toxicity, there are no effective treatments available which completely abolish its toxic effects. In MeHg poisoning, supportive care is given when necessary to maintain vital functions. In addition, the use of chelator agents assists the body's ability to eliminate mercury from the tissues. However, these drugs are of limited use because of their adverse side effects (Tchounwou et al 2003).

Some studies have focused their efforts on the protective effects of plants on diverse neuropathological conditions. In this regard, plants of the genus *Polygala* have been shown to possess protective effects against neuronal death and cognitive impairments in neurodegenerative disorders related to excitotoxicity (Park et al 2002; Lee et al 2004). In addition, preliminary data from our laboratory has demonstrated that *Polygala paniculata* extract causes a significant and dose-dependent inhibition of glutamate-induced nociception in mice, suggesting a possible 'anti-glutamatergic' effect.

Taking into account the absence of effective treatments for MeHg poisoning and that plants of the genus *Polygala* have been shown to possess beneficial effects in neuropathological conditions related to excitotoxicity, the aim of this study was to determine the possible in-vivo protective effects of *P. paniculata* extract against MeHg-induced neurotoxicity in mice. The activity of the antioxidant enzymes glutathione peroxidase (GPx) and glutathione reductase (GR), as well as the content of nonprotein thiols (NPSH) and thiobarbituric acid reactive substances (TBARS) were evaluated in mice cerebral cortex and cerebellum. The performance of the animals on the rotarod task was determined to detect possible motor deficits induced by exposure to MeHg.

Materials and Methods

Plant extract preparation

Polygala paniculata was collected in Rancho Queimado (Santa Catarina State, Brazil), and was classified by Dr Olavo de Araújo Guimarães (Federal University of Santa Catarina, Florianópolis, Brazil). The dried whole plant (500 g) was minced and extracted with 50% ethanol–water (1:3), while being stirred and macerated at room temperature (22 ± 3°C) for 14 days. The ethanol was evaporated and the extract (yield 135 g) was concentrated to the desired level and stored at −20°C until use. The extract was dissolved in 150 mM NaCl solution to the desired concentration just before use.

Phytochemical studies carried out by our group with *P. paniculata* extract (PE) have demonstrated the presence of many classes of constituents (Cristiano et al 2003). Using chemical and spectroscopic methods (EIMS, IR, ^{1}H and ^{13}C NMR, NOE difference spectroscopy), the structures of two xanthones (1-hydroxy-5-methoxy-2,3-methylenedioxyxanthone and 1,5-dihydroxy-2,3-dimethoxyxanthone) were determined, together with coumarin, murragatin and flavonol rutin. In addition, using gas chromatography coupled with mass spectrometry (HRGC-MS), it was possible to characterize two sterols (spinasterol and delta25-spinasterol) and the minor 1-hydroxy-2,3,5-trimethoxyxanthone.

Animals and treatment

Adult Swiss Albino male mice were bred in the animal facilities of the Federal University of Santa Catarina. The mice were maintained according to the Animal Care Guidelines from the National Institutes of Health of the United States of America, and all experiments were approved by our ethics committee for animal use (313/CEUA; 23080.026023/2004-39/UFSC). The animals were maintained at 23°C on a 12-h light/dark cycle with free access to food. The mice were separated in four experimental groups (control; MeHg; PE; and MeHg + PE) with seven animals each. The control group received tap water, which was freely available, and a 150 mM NaCl solution, by gavage ($10\,mL\,kg^{-1}$), twice a day. Groups MeHg and MeHg + PE were exposed to methylmercury (II) chloride (Sigma Chemical Co., St Louis, MO) based on a previous study from our laboratory ($40\,mg\,L^{-1}$ (Farina et al 2003b)). Groups PE and MeHg + PE received the *Polygala* extract solution ($100\,mg\,kg^{-1}$), diluted in 150 mM NaCl, by gavage ($10\,mL\,kg^{-1}$), twice a day. Tap water and a 150 mM NaCl solution were administered as vehicle in control conditions for MeHg and PE groups, respectively. Exposure was performed over two weeks, where liquid and solid ingestions were monitored daily.

Rotarod task and tissue preparation

The mice were subjected to the rotarod task, which was based on the study of Duham & Miya (1957), with minor modifications. Briefly, the homemade apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into four compartments by disks, 25 cm in diameter. The bar rotated at a constant speed of $17\,rev\,min^{-1}$. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for a period of 60 s. Before the beginning of treatments (phase 1) and after the treatment period (two weeks: phase 2), the selected animals were subjected to the rotarod task and the time of permanence on the apparatus was recorded. After the rotarod task, the animals were killed by decapitation and the brain cortex and cerebellum were quickly removed and placed on ice. The tissues were homogenized in HEPES 25 mM, pH 7.4 buffer (1:5, w/v) and rapidly centrifuged at 20 000 *g*, at 4°C for 30 min. The supernatants obtained were used for the determination of enzyme activity, the levels of nonprotein thiols (NPSH) and thiobarbituric acid reactive substances (TBARS).

Biochemical determinations

The activity of glutathione reductase and glutathione peroxidase was determined based on Carlberg & Mannervik (1985) and Wendel (1981), respectively. NPSH levels were evaluated based on Ellman (1959) and TBARS levels were evaluated based on Ohkawa et al (1979), with minor modifications (Farina et al 2003b). Protein concentration was determined according to Bradford (1976), using a bovine serum albumin as standard.

In-vitro experiments to detect possible chelating effects of *Polygala* extract

The possible chelating effects of the extract towards MeHg were performed based on the indirect determination of 'free' MeHg using reduced glutathione (GSH). Briefly, different concentrations of MeHg (0, 10, 25 50 and 100 μM) were incubated with GSH (50 μM) in the presence or in the absence of *Polygala* extract ($12.5\,\mu g\,mL^{-1}$) at 37°C (volume total of reaction = 1 mL). After incubation for 30 min, the amount of GSH remaining was determined (Ellman 1959), using 55′-dithiobis-(2-nitrobenzoic acid). The amount of *Polygala* extract added was equivalent to 50 nmol MeHg on a weight:weight base.

Statistical analysis

Statistical differences among groups were analysed by one-way analysis of variance followed by Duncan's multiple range test when appropriate. Differences were considered statistically significant when $P < 0.05$.

Results and Discussion

After two weeks of treatment, there were no differences in the body weight gain of the animals between groups. In addition, liquid and solid ingestion during treatments were not significantly different between groups (data not shown). A daily MeHg dose of 6.43 mg kg^{-1} body weight was calculated based on their daily liquid ingestion (6.1 mL/day).

MeHg exposure significantly decreased GPx activity in the brain cortex (40%) and cerebellum (30%). This phenomenon was prevented by administration of *Polygala* extract (Table 1). Conversely, MeHg exposure increased the activity of GR in the cerebral cortex (20%) and the cerebellum (34%), and *Polygala* extract abolished this effect in the cerebellum only (Table 1).

MeHg exposure also increased the levels of TBARS (Figure 1) in both encephalic structures (41% for cerebral cortex and 48% for cerebellum). Membranes were protected from MeHg-induced lipid peroxidation by administration of *Polygala* extract, as measured by TBARS.

No significant differences were observed between groups for NPSH levels in either of the encephalic structures (data not shown).

Table 2 depicts the performance of mice in the rotarod task before and after the two-week treatment. A marked deficit in the motor performance was observed for MeHg-exposed animals, which was abolished by administration of *Polygala* extract.

There are several studies showing that MeHg exposure causes deleterious neurotoxic effects in animals and man (Clarkson et al 2003). Although MeHg-induced neurotoxicity is a well described phenomenon, there are still no effective treatments available for MeHg poisoning. In fact, supportive care is given when necessary to maintain vital functions in MeHg poisoning and the treatment with chelator agents assists the body's ability to eliminate mercury from the tissues. However, these drugs are of limited use because of their adverse side effects (Tchounwou et al 2003). We have shown for the first time that *Polygala paniculata* extract possessed protective effects against MeHg-induced neurotoxicity in mice. Even though plants of the genus *Polygala* have been shown to possess protective effects against neuropathological conditions (Park et al 2002; Lee et al 2004), to the best of our knowledge, their beneficial effects against metal-induced neurotoxicity as yet have not been demonstrated.

Although some studies have pointed to the important pharmacological effects of extracts or isolated compounds

Table 1 Effects of MeHg and *Polygala paniculata* extract (PE) treatment on the activity of glutathione peroxidase and glutathione reductase from mice cerebral cortex and cerebellum

Enzyme activity	Control	MeHg	PE	MeHg + PE
Glutathione peroxidase (GPx)				
Cerebral cortex	23.5 ± 1.3^{a}	14.9 ± 0.7^{b}	24.7 ± 2.2^{a}	22.6 ± 0.9^{a}
Cerebellum	31.0 ± 2.6^{a}	21.4 ± 1.9^{b}	$27.8 \pm 3.9^{a,b}$	$27.3 \pm 2.5^{a,b}$
Glutathione reductase (GR)				
Cerebral cortex	33.5 ± 1.4^{a}	$41.5 \pm 1.4^{b,c}$	$38.9 \pm 3.5^{a,b}$	46.0 ± 1.9^{c}
Cerebellum	72.3 ± 1.6^{a}	99.8 ± 9.8^{b}	83.1 ± 4.1^{a}	77.1 ± 2.4^{a}

Data are expressed as mean ± s.e.m. (n = 7) and represented as mU mg^{-1}. Values not sharing the same letter, at the same row, are statistically different; $P < 0.05$.

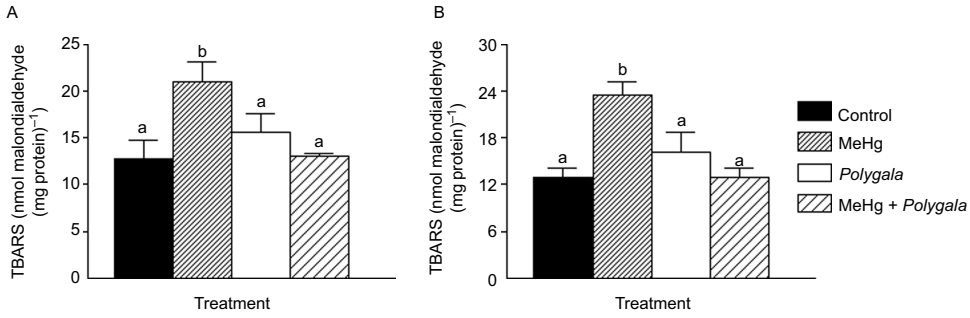

Figure 1 Effects of MeHg and *Polygala paniculata* extract treatments on the levels of TBARS from mice cerebral cortex (A) and cerebellum (B). Data are expressed as mean ± s.e.m. (n = 7). Values not sharing the same letter are statistically different; $P < 0.05$.

Table 2 Effects of MeHg and *Polygala paniculata* extract (PE) treatment on the performance of animals in the rotarod task

Task	Time on the rotarod (s)			
	Control	MeHg	PE	MeHg + PE
Phase 1	51.1 ± 5.4	60.0 ± 0	55.8 ± 4.1	53.4 ± 4.3
Phase 2	60.0 ± 0	32.3 ± 8.0*	60.0 ± 0	50.0 ± 6.7

Phase 1 and 2 represent the animals' time of permanence on the rotarod task obtained before the beginning of treatments and after the treatment period, respectively. Data are expressed as mean ± s.e.m. (n = 7) and represented as seconds. $*P < 0.05$, compared with control.

from plants of the genus *Polygala* (Park et al 2002; Lee et al 2004; Lin et al 2005), evidence of their possible adverse effects are scarce. In this regard, our experimental protocol detected no visible signs of toxicity in the *Polygala* extract-treated mice. In addition to the absence of changes in the body weight gain, it is noteworthy that a haematological analysis did not reveal any difference in the number of red and white blood cells in the extract-treated animals when compared with the control animals (data not shown).

The data showed a correlation between the pro-oxidant properties of MeHg and its neurotoxic effects. In fact, MeHg exposure decreased the activity of GPx in mouse cerebral cortex and cerebellum. Since GPx is crucial for the detoxification of endogenous peroxides, the levels of lipid peroxidation by-products (measured as TBARS) were significantly higher in the cerebral cortex and cerebellum of MeHg-exposed mice, which could have been due to decreased GPx activity, among other factors. These data indicated the occurrence of oxidative stress in the brain structures of MeHg-exposed mice. It was interesting to note that *Polygala* extract abolished both these alterations induced by MeHg, suggesting the potential relationship between them. In agreement with these findings, antioxidant compounds, such as xanthones and the flavonol rutin, have been identified in the chemical composition of *P. paniculata* (Cristiano et al 2003).

GR is an important enzyme involved in the reduction of glutathione disulfide (GSSG, also known as oxidized glutathione) to glutathione (GSH), using NADPH as a reducing cofactor (Gul et al 2000). Here, we showed that MeHg exposure increased GR activity in the cerebral cortex and cerebellum. Although studies on the effects of MeHg on GR activity are lacking in the literature, evidence shows that mercury is able to increase GR activity under in-vivo conditions (Lash & Zalups 1996). This increment could be related, at least in part, to the direct oxidative effects of mercury on endogenous GSH, which leads to an enhancement in GR activity. In fact, the increase in GR activity could be interpreted as a protective response to preserve the homeostasis of intracellular thiol status. It is important to state that, although it is well known that MeHg is able to react with and deplete thiol compounds, such as GSH, the levels of NPSH in mouse cerebral cortex and cerebellum remained unchanged after MeHg exposure. Furthermore, oxidative and xenobiotic insults can lead to an increase in GSH synthesis (Moskaug et al 2005), an effect that can mask thiol consumption by MeHg.

Although oxidative stress (Ou et al 1999) and the alteration of glutamate homeostasis (Aschner et al 2000; Farina et al 2003a; Manfroi et al 2004) are distinct mechanisms related to MeHg-induced neurotoxicity, they are closely related. In fact, the over activation of *N*-methyl-D-aspartate receptors (a glutamate receptor) leads to increased influx of Ca^{2+} ions (Choi 1992). Intracellular Ca^{2+} overload is associated with the generation of reactive oxygen species, which degrade cellular structural components (Lafon-Cazal et al 1993). Of particular importance, plants of the genus *Polygala* have been shown to possess protective effects against neuronal death and cognitive impairments in neurodegenerative disorders related to excitotoxicity (Lee et al 2004).

Even though the MeHg solutions were available 24 h per day for MeHg-exposed mice and *Polygala* extract was only administered twice a day by gavage during the treatment period, it could be possible that *Polygala* extract might have exerted its protective effects by chelating MeHg, preventing toxin absorption through the gastrointestinal tract. In this regard, in-vitro experiments were performed to detect the possible chelating effects of the extract toward MeHg. Table 3 shows that *Polygala* extract was unable to chelate MeHg. In fact, MeHg oxidized equimolar amounts of GSH, probably due to the formation of its glutathione S-conjugate (Yasutake et al 1989). This phenomenon was independent of extract addition to the reaction medium, indicating no chelating effects of the extract on the 'free' MeHg. These results reinforced the idea that the protective effects of *Polygala* extract were related to its antioxidant properties.

There is evidence that cerebellar cells are targeted selectively by mercury compounds in-vivo (Sanfeliu et al 2003) and that MeHg neurotoxicity affects the motor system (Grandjean et al 1997). In fact, the relationship between MeHg-induced motor deficit and MeHg-induced cerebellar damage is a well described phenomenon (Sakamoto

Table 3 In-vitro effects of *Polygala paniculata* extract (PE) on the MeHg-induced glutathione (GSH) oxidation

MeHg (nmol mL^{-1})	Without PE	With PE
0	50.0 ± 3.0	49.6 ± 3.5
10	43.6 ± 5.2	42.4 ± 2.3
25	27.8 ± 2.9	26.8 ± 2.9
50	0.38 ± 0.3	0.50 ± 0.1
100	0.34 ± 0.5	0.25 ± 0.2

Data are expressed as mean ± s.e.m. (n = 3) and represented as nmol of remaining reduced glutathione. The amount of added *Polygala* extract was equivalent to 50 μM MeHg on a weight:weight base. Analysis of variance indicated no significant differences between the conditions with and without *Polygala* extract.

et al 1993). In this regard, we have reported motor deficits in animals exposed to MeHg during adulthood (Dietrich et al 2005; Farina et al 2005) and the suckling period (Manfroi et al 2004). Here, we observed that MeHg exposure affected the motor performance of mice in the rotarod task after two weeks of treatment. Based on literature data, these results suggested that the harmful effect of MeHg on the motor performance was related, at least in part, to its deleterious pro-oxidative effects on mice cerebellum. Interestingly and similarly to oxidative stress in mice cerebellum, the behavioural/functional effect was abolished when MeHg was administered simultaneously with *Polygala* extract, suggesting a potential relationship between them.

In addition to MeHg, mercury vapour is also an important neurotoxicant responsible for many neurological signs observed in animals and man (Bittner et al 1998; Yoshida et al 2005). Taking into account that MeHg and mercury vapour have pro-oxidative properties in the mammalian brain, it could be possible that the neuroprotective effects of *Polygala* extract could be extended to intoxications involving mercury vapour. However, to confirm this hypothesis additional studies would be necessary.

Conclusions

The results provided evidence, for the first time, that *Polygala* extract exerted significant in-vivo protective effects against MeHg-induced neurotoxicity. Although to date its precise site of action remains unclear, the antioxidant properties of *Polygala* extract constituents are likely to be responsible for the greater part of its neuroproective effects.

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