Acta Scientiarum
http://periodicos.uem.br/ojs
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v46i1.69047

Effects of curcumin and fish oil on motor behavior and biochemical parameters in mice exposed to rotenone

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ABSTRACT. Parkinson's disease is characterized by dopaminergic neuronal loss, mainly in the substantia nigra pars compacta. Currently, pharmacological treatment reduces the symptoms of the disease, however it does not affect its progression, and often leads to significant adverse effects. In this context, natural products have been explored as a potential source of neuroprotective substances. In this study, the neuroprotective potential of curcumin (CUR) and fish oil (FO) was evaluated against rotenone, which serves as a chemically induced experimental PD model. Male Swiss mice were treated with CUR, FO, CUR + FO or vehicle for seven days. On the eighth day, the animals began receiving rotenone in addition to the treatments for 30 days. At the end of the treatment, locomotor function, oxidative stress and toxicological markers were evaluated. Regarding motor parameters, CUR was able to reduce the number of slippages and bradykinesia in animals treated with rotenone. FO also improved motor coordination, but no additional benefits were observed when FO and CUR were associated. Interestingly, FO increased brain lipid peroxidation, which was attenuated when mice were treated with CUR, suggesting a beneficial effect of the association. In conclusion, CUR and FO possess protective properties against rotenone-induced neurotoxicity. However, the potential benefits of their combined use in relation to PD still require further studies. For example, investigating different doses and treatment durations, as well as evaluating parameters that assess neurodegeneration.

Keywords: Parkinsonism; omega-3 polyunsaturated fatty acids; oxidative stress; curcumin; neuroprotection.

Received on June 25, 2023.
Accepted on March 15, 2024.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). The ventrolateral layer of the SNpc, where the neurons project to the striatum, is primarily affected (Kalia & Lang, 2015). Neuronal loss occurs due to the accumulation of insoluble α-synuclein protein, through intracellular cytoplasmic inclusions that form the Lewy bodies, which are the main histopathological hallmark of PD (Goedert, Spillantini, Del Tredici, & Braak, 2013). In addition, other pathological features such as the occurrence of oxidative stress, mitochondrial dysfunction and neuro inflammation are involved in dopaminergic neurodegeneration (Raza, Anjum, & Shakeel, 2019). Motor symptoms, such as bradykinesia, muscle stiffness and tremors usually appear when 70-80% of dopaminergic neurons are damaged (Poewe et al., 2017).

The pharmacological treatment of PD does not alter the progression of the disease, it only enhances motor control in the early stages. Current drugs work by elevating intracerebral dopamine concentrations or by stimulating dopamine receptors. Levodopa is the most commonly prescribed drug and provides the most significant symptomatic relief. However, over the long term this drug can lead to adverse effects, such as dyskinesia (Kalia & Lang, 2015).

Due to the undesirable effects of existing long-term treatments several studies have focused on the search for neuroprotective substances. These compounds have the potential to alter the course of the disease by

preventing dopaminergic neurodegeneration. In this context, natural products, such as phenolic compounds and marine derivatives, have been investigated as therapeutic adjuvants for PD due to their biological and pharmacological activities (Huang, Zhang, & Cui, 2019; Kujawska & Jodynis-Liebert, 2018).

A widely studied polyphenol is curcumin (CUR), a spice used in cooking, derived from the rhizome of *Curcuma longa* (saffron), which has been studied as a neuroprotective agent (Wu et al., 2015). Curcumin has antioxidant, anti-inflammatory and anti-apoptotic properties (He, Uchida, Megumi, Tsuge, & Nakayama, 2015; Ramkumar et al., 2018; Wang et al., 2017). Moreover, it is a lipophilic molecule that capable of crossing the blood-brain barrier and acting within the central nervous system, allowing its use as a neuroprotector, for example against PD (Patel et al., 2022).

The high levels of reactive species formed in PD and the downregulation of antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx), can play a fundamental role in the dopaminergic damage (Islam, 2017). Previous studies have shown that CUR mitigates motor dysfunction and improve the redox status, through reduction of oxidative damage to macromolecules or enhancing antioxidant defenses, in rodents subjected to experimental induced parkinsonism (Khatri & Juvekar, 2016; Ramires Júnior et al., 2021; Song, Nie, Li, & Du, 2016). Furthermore, CUR also protects mitochondrial dysfunctions and restores complex I function in experimental models of PD (Mythri, Harish, Dubey, Misra, & Srinivas Bharath, 2011; Ramires Júnior et al., 2021).

Other widely studied compounds with neuroprotective potential are omega-3 polyunsaturated fatty acids (ω-3 PUFAs). They are mainly composed of α-linolenic acid, stearidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA are important components of cell membranes, aiding in maintaining their structure, permeability, and fluidity (Shahidi & Ambigaipalan, 2018). They are essential nutrients obtained from the diet and are primarily found in fish, olive and linseed oils, and also in algae e microalgae biomass (McAnulty et al., 2010; Rismani & Shariati, 2017). The beneficial properties of ω-3 PUFAs have been previously described for several pathologies, including neurodegenerative diseases. (Healy-Stoffel & Levant, 2018; Li & Song, 2020). Previous studies, have also demonstrate that ω-3 PUFAs improve motor impairments, reduces oxidative stress, attenuate inflammation, and protect against dopaminergic damage in experimental models of parkinsonism (Coulombe et al., 2016; Hernando et al., 2019; Ji et al., 2012).

In the brain, ω-3 PUFAs, such as DHA, can be oxidated generating reactive products, such as aldehydes that damage biomolecules (Appolinário, Derogis, Yamaguti, & Miyamoto, 2011) however, when combined with antioxidants, this oxidation tends to be reduced. Studies have already shown that the combination of fish oil (FO) with phenolic compounds, such as quercetin and green tea derivatives, provides neuroprotection against brain damage (Denny Joseph & Muralidhara, 2013, 2012). Despite the evidence in the literature demonstrating the potential of substances, such as curcumin and ω-3 PUFAs in PD, there are no studies evaluating the effect of their association in experimental models of the disease. In this context, the aim of this study was to evaluate the effect of CUR and FO, as well as their combination, against the neurotoxicity induced by rotenone in mice, while evaluating behavioral and biochemical parameters.

Material and methods

Chemicals

Curcumin, rotenone, nicotinamide phosphate adenine dinucleotide, nicotinamide adenine dinucleotide and malondialdehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fish oil was obtained from a commercial brand in the form of oily capsules (Vitamed Ltda, RS, Brazil). All the reagents used in the experiments were of analytical grade.

Animals

Male Swiss mice, 3 months old (40-50 g) were kept at 22 ± 2 °C under a 12-hour light/12-hour dark cycle and provided with commercial pelletized rodent feed containing carbohydrates (63.4%), proteins (25.9%) and lipids (10.6%) (Nuvilab, Quimtia, Brazil), and water *ad libitum*. The experiments were carried out after approval of the protocol by the Institutional Ethics Committee (approval number 23116.004990/2015-48 - Pq056-2015) and in accordance with the guidelines of the Brazilian National Council for Animal Experimentation Control (CONCEA).

Experimental design

The animals were randomly divided into 5 experimental groups, each containing 10 animals, as described in Table 1. On day 0 (day zero), all animals were weighed and underwent training on the Beam Walking and Pole Test apparatus (described below). The solution containing CUR was prepared daily, solubilizing CUR in soy oil and sonicated. On the first day, at the same time each day, the animals received oral administrations via gavage containing soy oil (control), CUR, FO or CUR + FO, once a day, for 7 days, as a pre-treatment (Denny Joseph & Muralidhara, 2012). On the eighth day (day 8), intraperitoneal administration of rotenone (ROT) started 1 hour after the oral treatments. All treatments remained for 30 days. The ROT solution was prepared as described by Cannon et al. (Cannon et al., 2009). The animals were weighed every three days and the doses were readjusted if necessary. On the 29th and 30th, behavioral tests Open Field, Beam Walking and Pole Test were performed (Figure 1). On day 31, the animals were euthanized with sodium thiopental (75 mg kg^{-1}) plus lidocaine (10 mg kg^{-1}) and a cardiac puncture was performed. Brain tissue samples (right and left hemispheres) were collected in a cold Petri dish and stored at -80 °C for further biochemical analysis.

Table 1. Experimental groups.

Group (n = 10)	Treatment	
	Oral pathway	Intraperitoneal pathway
CONT	Soy Oil	2% DMSO and 98% MCT
ROT	Soy Oil	ROT (1 mg kg^{-1})
CUR + ROT	CUR (50 mg kg^{-1})	ROT (1 mg kg^{-1})
FO + ROT	FO (300 mg kg^{-1} DHA and 450 mg kg^{-1} EPA)	ROT (1 mg kg^{-1})
CUR + FO + ROT	CUR (50 mg kg^{-1}) + FO (300 mg kg^{-1} DHA and 450 mg kg^{-1} EPA)	ROT (1 mg kg^{-1})

CONT (control group); CUR (curcumin); FO (fish oil); DHA (docosahexaenoic acid); EPA (eicosapentaenoic acid); ROT (rotenone); DMSO (Dimethylsulfoxide); MCT (medium chain triglycerides).

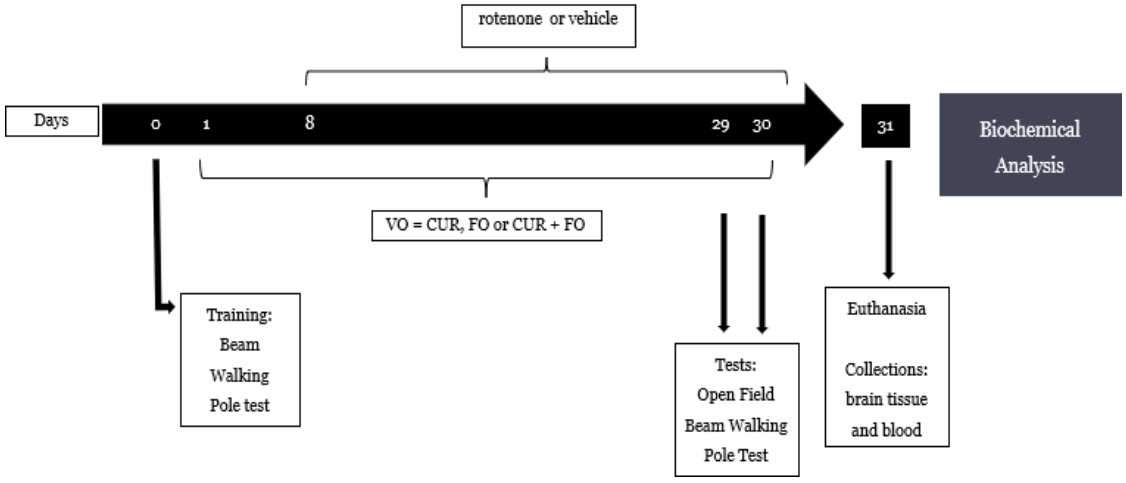

Figure 1. Experimental protocol. CUR (curcumin); FO (fish oil); CUR + FO (curcumin + fish oil).

Behavioral tests

Foot fault test

The Foot Fault Test assesses the sensorimotor coordination through the failure of the rear during spontaneous locomotion. Mice were placed on a high grid, measuring 30 L x 35 W x 31 H cm, with each grid opening measuring 2.5 cm^2. An evaluator filmed the animals for 5 minutes, during which the number of rear leg failures was counted (Zhang et al., 2019).

Open field test

The Open Field assesses the animal's spontaneous locomotor and exploratory activities. The equipment consists of a wooden box measuring 30 L x 22 W and 30 H cm. Each animal was placed in the center of the box for 5 minutes and the total distance covered, and the number of rearing were measured (Tatem et al., 2014). The parameters were recorded and analyzed in a video program (SMART Video Tracking System - Panlab Harvard Apparatus, Spain).

Beam walking test

Beam Walking is used to assess the balance and motor coordination of animals. The equipment consists of a wooden beam 100 cm long and 12 mm wide, 50 cm high from the floor, tilted at an angle of 15 degrees. At the end of the beam, there is a dark box containing sawdust and food. Prior to training, each animal was acclimatized in the box for 2 minutes and the animals were placed in the equipment 1 day before the start of treatment (day 0). The training was carried out through 5 attempts to cross the beam, in which he was taught to cross the box with sawdust. On the 30th, the animals were submitted to the test, where the crossing time (maximum of 1 minute) and the number of times that one of the posterior members slipped on the beam was recorded (Colle et al., 2020).

Pole test

The Pole Test is used to measure bradykinesia in animals. The equipment consists of a vertical wooden pole (50 cm long x 0.8 cm in diameter), wrapped in tape to increase traction, positioned on a horizontal support. On day 0, the animals underwent training on the equipment. The animals were positioned upside down on the pole type and the latency of descent was measured, with a maximum of 60 seconds to perform the descent. The training sessions were carried out 3 times. On the 30th, the test was performed, during which the animals were placed upside down on the top of the mast, and the time to turn their heads and the time to descend to the ground were measured (Colle et al., 2020; Park, Park, Yang, & Oh, 2013).

Biochemical analysis

Tissue preparation

The right brain samples (n = 7 animals/group) were homogenized in 100 mM Tris-HCl buffer, 2 mM EDTA and 5 mM $MgCl_2$ $6H_2O$ (1:10, w/v), pH 7.75, and centrifuged at 5000 xg at 4 °C for 10 min. The supernatant was used to determine the levels of substances reactive to thiobarbituric acid (TBARS) and the content of non-protein thiol (NPSH). For the measurement of catalase activity (CAT), the same homogenate was centrifuged at 16,000 xg for 20 min at 4 °C.

Non-protein thiol (NPSH) content

The content of NPSH was measured using the method described by Ellman (Ellman, 1959). The samples were precipitated in 10% trichloroacetic acid and centrifuged at 5,000 xg for 10 min. The supernatant was incubated with 10 mM DTNB in 1M phosphate buffer at pH 7.0. Absorbance was measured at 412 nm (ELX Microplate Reader, Biotek Instruments, Vermont, USA) and a GSH curve was used as a standard. The data were expressed as nmol NPSH/mg protein.

Lipid peroxidation

Lipid peroxidation was evaluated by substances reactive to thiobarbituric acid (TBARS), as described by Oakes and Van Der Kraak (2003), with some modifications. In brief, the samples were incubated with 20% acetic acid, 0.8% thiobarbituric acid, water and 8.1% SDS and heated to 95 °C for 30 min. Subsequently, 100 µL of water and 500 µL of n-butanol were added and the mixture was centrifuged at 5000 xg for 10 min at 4 °C. The fluorescence of the organic phase was measured at an excitation of 515 nm and an emission of 553 nm, using a fluorometer (FilterMax F5-Multi-Mode Microplate Reader, Molecular Devices, CA, USA). The results were calculated using a malondialdehyde (MDA) curve and expressed as nmol MDA/mg protein.

Catalase

Catalase activity (CAT) was measured using the method from Aebi et al. (Aebi, Wyss, Scherz, & Skavril, 1974). The consumption rate of H_2O_2 was measured spectrophotometrically at 240 nm (UV Vis spectrophotometer, Perkin Elmer, Massachusetts, USA). The results were expressed as µmol H_2O_2 consumed/min/mg of protein.

Protein determination

Protein content was measured using the method described by Lowry et al. (Lowry, Rosenbrough, Farr, & Randall, 1951), using bovine albumin as standard.

Measurement of liver transaminases and creatinine

Blood samples were centrifuged at 1000 xg for 10 min at room temperature, and the serum was separated for further analysis. Hepatic transaminases (ALT - alanine aminotransferase, and AST - aspartate aminotransferase) and creatinine levels were measured by enzymatic assays, using diagnostic kits following the manufacturers' instructions (AST and ALT, Biotécnica Biotecnologia Avançada, Varginha, MG, Brazil; creatinine, Labtest Diagnostica, Lagoa Santa, MG, Brazil).

Statistical analysis

The results are expressed as mean ± standard error of the mean (S.E.M). Data from all experiments were tested for normality using the Kolmogorov - Smirnov test. The Grubs test with a significance level of 0.05% was applied for the analysis of outliers. Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by the Bonferroni's or Newman-Keuls *post hoc* test. P-values less than 0.05 were considered statistically significant. Statistical analyzes and all graphs were performed using GraphPad Prism (GraphPad Software, version 8.0, San Diego, CA, USA).

Results

Behavioral tests

In this study, we evaluated the effects of the treatments in motor behavior in mice using different tests. In the foot fault, the animals exposed to ROT showed a higher number of failures in the hind legs compared to the control group ($p<0.05$; Figure 2A), suggesting an impairment in the locomotor function. However, in the open field, ROT was not capable of modify the spontaneous locomotor activity (distance traveled) or exploratory behavior (number of rearing) when compared to control group (Figure 2B and C). Considering the treatments, FO, CUR and the association, did not have any effect on the motor parameters analyzed (Figure 2).

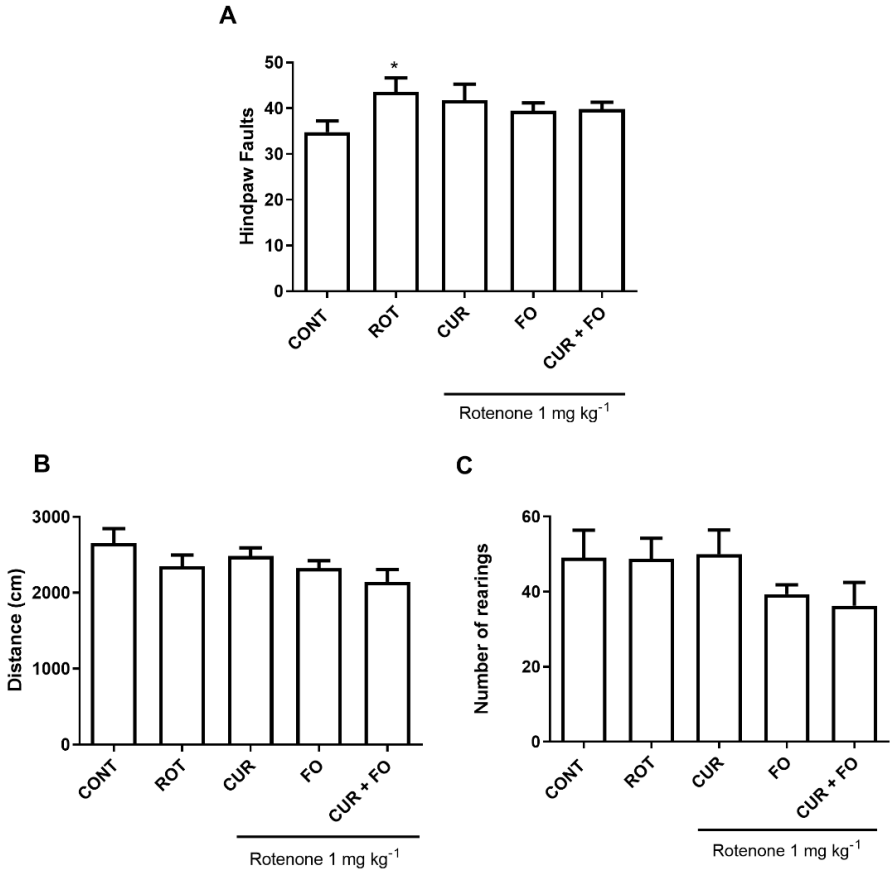

Figure 2. The effects of curcumin (CUR 50 mg kg^{-1}), fish oil (FO; DHA 300 mg kg^{-1} and EPA 450 mg kg^{-1}) and CUR+ FO on the number of (A) hindpaw faults in the foot fault test (A), and in (A) spontaneous locomotor (distance) and (B) exploratory (number of rearings) activities in the open field test. in mice exposed to rotenone (ROT, 1 mg kg^{-1}). Results are expressed as mean ± standard error of mean (S.E.M) of 9-10 animals/group. *p<0.05 compared to the control group (CONT) (one-way ANOVA followed by the Bonferroni's *post hoc* test).

The beam walking test evaluates the balance and motor coordination of the animals through parameters slip steps and crossing time. As shown in the Figure 3, ROT increases the number of slip steps when compared to control group, indicating a deficit in motor skills and balance. Interestingly, CUR ($p<0.01$), FO and CUR + FO ($p<0.001$) reduced this parameter when compared to ROT group, indicating an improvement in balance. Regarding crossing time, no differences were observed between the experimental groups.

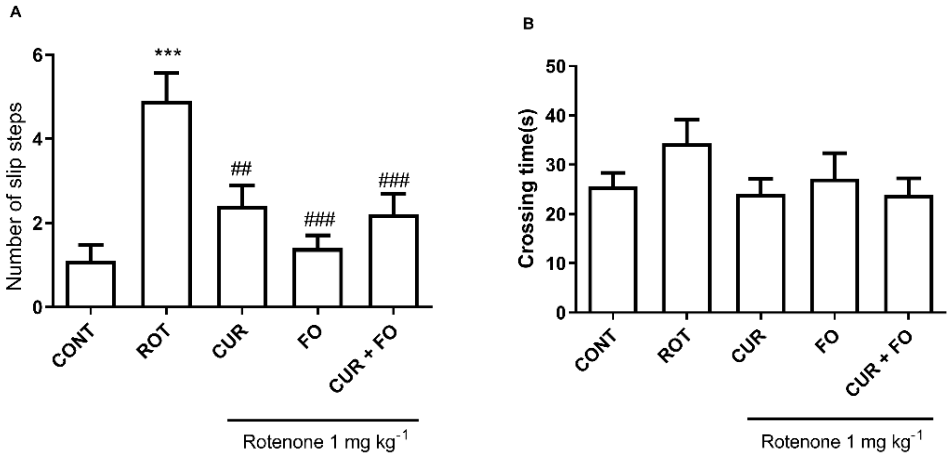

Figure 3. The effects of curcumin (CUR 50 mg kg^{-1}), fish oil (FO; DHA 300 mg kg^{-1} and EPA 450 mg kg^{-1}) and CUR+ FO in (A) number of slip steps and (B) crossing time in the beam walking test, in mice exposed to rotenone (ROT, 1 mg kg^{-1}). Results are expressed as mean ± S.E.M of 10 animals/group. ***p<0.001 compared to the control group (CONT); ##p<0.01; ###p<0.001 compared to ROT group (one-way ANOVA followed by the Bonferroni's *post hoc* test).

Bradykinesia, one of the characteristic symptoms of PD, was evaluated by the pole test. As expected, ROT increases the latency time to initiate the movement and also the descent time in the apparatus when compared to control mice ($p<0.05$). Treatment with CUR prevented the effects ROT in this parameter ($p<0.01$) while FO and the association of compounds did not present statistical difference from ROT group (Figure 4).

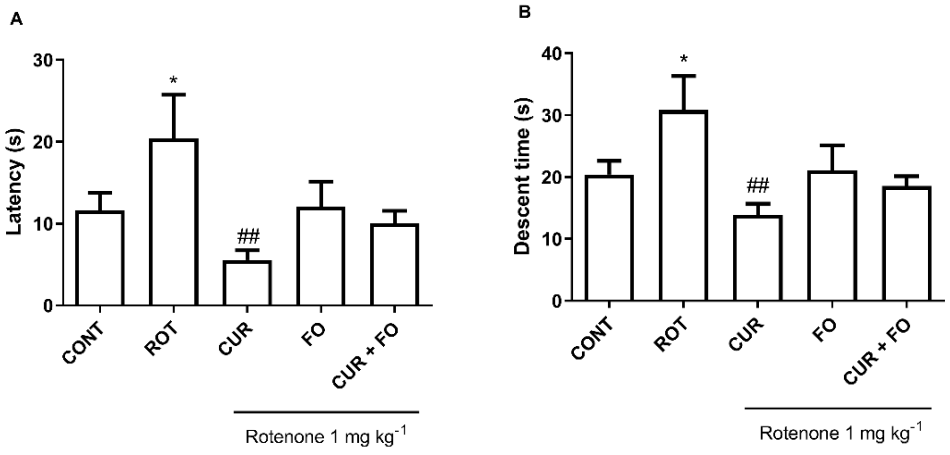

Figure 4. The effects of curcumin (CUR 50 mg kg^{-1}), fish oil (FO; DHA 300 mg kg^{-1} and EPA 450 mg kg^{-1}) and CUR+ FO in (A) latency and (B) descent time in the pole test, in mice exposed to rotenone (ROT, 1 mg kg^{-1}). Results are expressed as mean ± S.E.M of 9-10 animals/group. *p<0.05 compared to the control group (CONT); ##p<0.01 compared to ROT group (one-way ANOVA followed by the Bonferroni's *post hoc* test).

Biochemical analysis

Since PD is associated with the presence of oxidative stress, we evaluated the effects of treatments on the content of NPSH, lipid peroxidation (TBARS) and catalase enzyme activity. Our results showed that ROT did not modify oxidative stress parameters evaluated, however mice treated with FO presented a significant increase in levels of lipid peroxidation compared to the control group ($p<0.05$). Interestingly, the CUR + FO group regulated these levels close to the control group (Figure 5). Additionally, as observed in Table 2, there was no significant difference between the experimental groups in AST, ALT and creatinine levels, indicating that the treatments did not cause hepatic and renal damage.

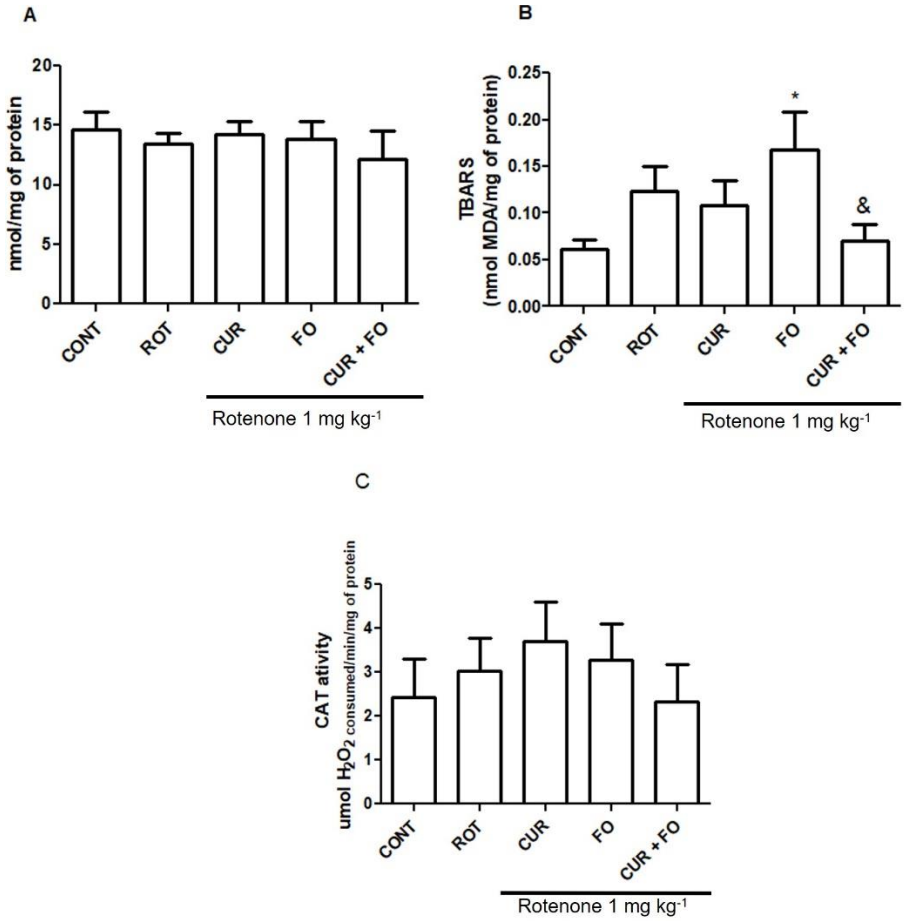

Figure 5. The effects of curcumin (CUR 50 mg kg^{-1}), fish oil (FO; DHA 300 mg kg^{-1} and EPA 450 mg kg^{-1}) and CUR+ FO in (A) non-protein thiols (NPSH), (B) thiobarbituric acid reactive substances (TBARS) and (C) catalase activity in brain tissue of mice exposed to rotenone (ROT, 1 mg kg^{-1}). Results are expressed as mean ± S.E.M of 5-7 animals/group. *p<0.05 compared to the control group (CONT); &p<0.05 compared to FO group (one-way ANOVA followed by the Newman-Keuls *post hoc* test).

Table 2. The effects of curcumin (CUR 50 mg kg^{-1}), fish oil (FO; DHA 300 mg kg^{-1} and EPA 450 mg kg^{-1}) and CUR+ FO on hepatic transaminases (AST and ALT) and creatinine of mice exposed to rotenone (ROT, 1 mg kg^{-1}).

Groups	ALT (U L^{-1})	AST (U L^{-1})	CRE (mg dL^{-1})
CONT	37.57 ± 6.33	198.80 ± 31.17	0.40 ± 0.07
ROT	40.43 ± 8.74	214.50 ± 33.01	0.35 ± 0.05
CUR	28.71 ± 14.77	148.40 ± 37.55	0.45 ± 0.07
FO	35.67 ± 6.44	246.90 ± 51.25	0.36 ± 0.06
CUR + FO	26.29 ± 6.26	210.70 ± 35.79	0.39 ± 0.06

ALT (alanine aminotransferase), AST (aspartate aminotransferase), CONT (control group); CRE (creatinine), CUR (curcumin); FO (fish oil); ROT (rotenone); DMSO (Dimethylsulfoxide); MCT (medium chain triglycerides). Results are expressed as mean ± S.E.M of 7-10 animals $group^{-1}$.

Discussion

Neuroprotective strategies have been extensively studied for their potential in preventing and/or treating neurodegenerative diseases, like PD. Among these, natural products, such as CUR and oils rich in ω-3 PUFAs, have shown promise. In this study, we investigated the effects of these compounds and its association in an *in vivo* experimental model of PD.

Several experimental models have been used to study the physiopathology of PD and to develop new drugs. In our study, we choose ROT-induced model, which replicates motor and biochemical characteristics of PD in mice, through the inhibition of the mitochondrial complex I activity and dopaminergic neuronal damage (Radad et al., 2019). Here, we observed characteristic behavioral alterations in mice exposed to ROT, including impaired motor coordination and balance, delayed movement initiation and gait abnormalities, corroborating previous literature data (Gokul & Muralidhara, 2014; Kundu, Das, Tripathy, & Sahoo, 2016; Ramires Júnior et al., 2021).

The neuroprotective effects of CUR against neurotoxins induced parkinsonism have been documented in the literature (Chakraborty, Karmenyan, Tsai, & Chiou, 2017; Darbinyan et al., 2017; Ramires Júnior et al.,

2021). In our behavioral analysis, we observed that CUR prevented the motor damage induced by rotenone. This was evident in the foot fault, which evaluates motor coordination and balance, and pole test, which evaluates bradykinesia. The ability of CUR to mitigate motor dysfunction may be associated to its capacity to reduce the dopaminergic damage induced by ROT. Cui, Li, and Zhu, (2016) demonstrated that CUR induces an increase in tyrosine hydroxylase (TH) activity, a marker of dopaminergic neurons, in the SNPc of ROT injured rats. Moreover, anti-inflammatory and antioxidant properties of CUR can be described to contribute to its effectiveness against experimental PD (Khatri & Juvekar, 2016; Sharma, Sharma, & Nehru, 2017).

Similarly, FO protected against neurotoxicity induced by rotenone, as can be seen in the reduction of gait abnormalities compared to untreated animals. Previous studies have shown that oils rich in ω-3 PUFAs have the ability to increase dopamine levels and its metabolites (Barros et al., 2017), as well as the levels of dopamine receptors and tyrosine hydroxylase (Wang et al., 2018a), demonstrating a direct effect on the modulation of dopaminergic neurons. The neuroprotective effects of ω-3 PUFAs can also be attributed to its capacity to reduce the neuroinflammation and oxidative stress in different experimental models of parkinsonism in rodents (Denny Joseph & Muralidhara, 2012; Ji et al., 2012; Ortiz et al., 2013; Wang et al., 2018b)

While several studies have been shown that fish oil and ω-3 PUFAs act as antioxidants (Barros et al., 2017; Denny Joseph & Muralidhara, 2012; Muntané et al., 2010), other studies have indicated that these compounds increase the lipid peroxidation in brain tissues (Kabuto, Amakawa, Mankura, Yamanushi, & Mori, 2009; Naudí et al., 2017). This can be explained by the high content of ω-3 PUFAs in cellular membranes making them more susceptible to oxidation by reactive species, due to the presence of double bonds in side chains (Naudí et al., 2017; Song & Miyazawa, 2001). Corroborating, our results evidenced an increase in lipid peroxidation after the treatment with FO compared to control group. Interestingly, when CUR was associated with FO, this increase did not occur. This suggests that the antioxidant properties of curcumin may help to prevent the peroxidation of PUFAs in the brain overall. The benefits of combining FO with other phenolic compounds was also previously demonstrated (Denny Joseph & Muralidhara, 2013, 2012). Furthermore, we evaluated antioxidant defenses, such as NPSH (mostly GSH) content and CAT activity, however FO and CUR cannot modify these biomarkers. By the end, it is important to highlight that ROT was not able to modify oxidative stress parameters compared to control group, however the locomotor impairment was evident. Regarding toxicity parameters, no changes were observed in markers of liver and kidney function at the end of treatment.

Conclusion

In summary, our findings suggest that both FO and CUR possess neuroprotective effects in rotenone-induced parkinsonism in mice, however, no improvement of the effects were observed when they were associated. Moreover, FO lead to increase the lipid peroxidation and, which was attenuated when CUR was administered concurrently, indicating an antioxidant effect of CUR. Finally, we believe the use of phenolic antioxidants along with ω-3 PUFAs may indeed be interesting in the prevention/treatment of PD. However, further studies are needed to optimize dosing and treatment duration, and also evaluate other pharmacological parameters, such as specific markers of dopaminergic neurodegeneration.

Acknowledgments

This study was financed in part by the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) - Finance Code 001 and by *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq, research grant 449650/2014-6 and 309840/2022-8).

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