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# EFEITOS DE N-ACETILCISTEÍNA EM UM MODELO DE DOENÇA DE PARKINSON EM LARVAS DE PEIXE-ZEBRA

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Dissertação apresentada ao Programa de Pós-Graduação em Neurociências do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de mestre(a) em Neurociências.

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**ABSTRACT**

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting on average 2-3% of the individuals older than 65 years. In addition to its highly debilitating motor symptoms, non-motor symptoms may precede by many years their motor counterparts, which may characterize a prodromal phase of PD. A potential pharmacological strategy is to introduce neuroprotective agents at an earlier stage in order to prevent further neuronal death. N-acetylcysteine (NAC) is an antioxidant, anti-inflammatory and glutamatergic modulator that has shown various therapeutic benefits in brain disorders. In this study, it was evaluated the effect of NAC to prevent the damage induced by 6-OHDA on motor, cognitive and morphological parameters in a PD model in larval zebrafish. NAC was able to prevent the motor and cognitive deficits and morphological alterations caused by exposure to 6-OHDA, which reinforce the relevance of its neuroprotective effects. By acting on different relevant targets in the pathophysiology of PD, NAC is a potential candidate for prevention and treatment of PD.

**RESUMO**

A Doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum, afetando em média 2-3% dos indivíduos com idade superior a 65 anos. Além dos sintomas motores altamente debilitantes, os sintomas não-motores podem preceder em muitos anos o aparecimento dos sintomas motores, podendo caracterizar uma fase prodrômica da DP. Uma potencial estratégia farmacológica é a introdução de agentes neuroprotetores no estágio inicial da doença, a fim de prevenir a morte neuronal decorrente do progresso da neurodegeneração. A N-acetilcisteína (NAC) é um antioxidante, anti-inflamatório e modulador glutamatérgico que mostrou vários benefícios terapêuticos em transtornos psiquiátricos. Neste estudo, foi avaliado o efeito da NAC na prevenção dos danos induzidos por 6-Hidroxidopamina (6-OHDA) sobre parâmetros motores, cognitivos e morfológicos em um modelo de DP em larvas de peixes-zebra. Nossos resultados mostraram que NAC foi capaz de prevenir os déficits motores e cognitivos e as alterações morfológicas causadas pela exposição a 6-OHDA, o que reforça a relevância de seus efeitos neuroprotetores. Por atuar em diferentes alvos relevantes na fisiopatologia da DP, NAC é um potencial candidato para prevenção e tratamento de DP.

**LISTA DE ABREVIATURAS**

**ATP:** Trifosfato de adenosina

**DAT:** Transportador de dopamina

**$H_2$DCFDA :** 2',7'-dichlorodiidrofluoresceina diacetato

**DP**: Doença de Parkinson

**DPOC:** Doença Pulmonar Obstrutiva Crônica

**EROs:** Espécies Reativas de Oxigênio

**GSH:** Glutationa

**IL-6:** Interleucina 6

**IL-1β:** interleucina 1β

**MPTP**: 1-metil-4-fenil-1,2,3,6-tetraidropiridina

**NAC:** N-acetilcisteína

**TH:** Tirosina Hidroxilase

**TNF-α:** Fator de Necrose Tumoral Alfa

**6-OHDA**: 6-Hidroxidopamina

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*“Na vida, não existe nada a temer, mas a entender.”*

*Marie Skłodowska Curie*

**SUMÁRIO**

## 1. INTRODUÇÃO

### 1.1. Doença de Parkinson

A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum e afeta 0,3% da população mundial e cerca de 3% da população de indivíduos com mais de 80 anos de idade (Poewe et al., 2017). O aspecto fisiopatológico da DP é caracterizado pela presença de corpos de *Lewy* e por degeneração progressiva de neurônios dopaminérgicos que se projetam da parte compacta da substância negra ao estriado (Poewe et al., 2017). A morte dos neurônios dessas vias, e consequente diminuição da liberação de dopamina nessas áreas, é considerada a causa das diversas anormalidades de movimento observadas nessa patologia (Kalia & Lang, 2015).

A etiologia da DP não está completamente esclarecida, porém sabe-se que possui perfil multifatorial, sendo assim, diversos fatores contribuem juntamente para seu surgimento e progresso. Estudos demonstram o aumento do estresse oxidativo (Yan, Wang & Zhu, 2013), diminuição do aporte de glutationa (GSH) (Smeyne & Smeyne, 2013), neuroinflamação (Niranjan, 2014), disfunções mitocondriais (Subramaniam & Chesselet, 2013) e alteração de genes como parkina, PINK1, DJ-1 e LRRK2 (Dexter & Jenner, 2013) como tendo um papel chave na etiologia da DP. Também foram observadas disfunções de sistemas importantes na degradação de proteínas disfuncionais, como mecanismos de autofagia lisossomal (Michel, Hirsch & Hunot, 2016) e o sistema ubiquitina-proteossoma (Rott et al., 2014). Disfunções nesses sistemas levam a um aumento de proteínas inapropriadamente dobradas em especial a α-sinucleína, e consequente formação de massas de agregados proteicos neurotóxicos como os corpos de *Lewy* (Poewe et al., 2017).

Os principais sintomas motores da DP são caracterizados por dificuldade na iniciação do movimento (acinesia), redução da amplitude e da velocidade dos movimentos voluntários (bradicinesia), tremor de 4 a 6 Hz em repouso, rigidez muscular (resistência aumentada a deslocamentos passivos) e postura flexionada (Kalia & Lang, 2015). Esses sintomas pioram progressivamente conforme o avanço da doença. Apesar de ser conhecida como um distúrbio do movimento, a DP apresenta uma gama de sintomas igualmente debilitantes conhecidos como não-motores, caracterizados por distúrbios gastrointestinais, autonômicos, cognitivos e de humor

(Dexter & Jenner, 2013). Os sintomas não-motores têm chamado especial atenção nos últimos anos pois podem preceder em muitos anos os sintomas motores, caracterizando uma fase prodrômica da DP (Pellicano et al., 2007; Berg et al., 2012). Além disso, existe alta comorbidade da DP com transtornos psiquiátricos, incluindo transtornos de ansiedade, depressão e insônia (Kalia & Lang, 2015). Em especial, os transtornos de ansiedade possuem um grande impacto no prognóstico da DP, estando relacionados com piora dos sintomas cognitivos e menor qualidade de vida (Chen & Marsh, 2014).

Os fármacos utilizados atualmente para o tratamento da DP tem como objetivo aumentar a neurotransmissão dopaminérgica, aliviando os sintomas motores, entretanto, nenhum deles é capaz de modificar a evolução da doença (Kalia & Lang, 2015). A levodopa é um análogo da dopamina que é convertido em dopamina tanto na periferia quanto no sistema nervoso central e é administrada juntamente com carbodopa, que previne a conversão periférica de levodopa (Johnson, 2015). Devido ao seu baixo custo e sua alta eficácia inicial, se tornou a escolha de tratamento mais comum. Sua principal desvantagem é o aumento gradual das flutuações motoras, que começam a aparecer depois de 4 a 5 anos de tratamento (Johnson, 2015). Além disso, a levodopa pode causar discinesia e agravamento de sintomas em pacientes que possuem transtornos neuropsiquiátricos, incluindo alucinações e psicose (Johnson, 2015). Outra grande limitação clínica é que nenhum dos fármacos disponíveis é capaz de tratar os sintomas não-motores da DP (Kalia & Lang, 2015).

Uma possível explicação para a ineficácia relativa de tais tratamentos está relacionada ao atraso de seu início, que geralmente começa com o aparecimento dos sintomas motores, quando a morte neuronal está em estágio avançado (Berg et al., 2012). Portanto, uma estratégia farmacológica potencial seria identificar os indivíduos de risco durante a fase prodrômica da doença, e introduzir agentes neuroprotetores durante esse estágio inicial, a fim de evitar que ocorra uma maior morte neuronal (Dexter & Jenner, 2013).

### 1.2. N-acetilcisteína

*Drug repurposing* é um termo utilizado para descrever o reposicionamento de compostos farmacológicos conhecidos e comercializados, para atingir novos propósitos terapêuticos. É uma estratégia atraente para o desenvolvimento de medicamentos devido às economias de pesquisa, financiamento e tempo (Insel et al., 2013; Langedijk et al., 2015; Klug, Gelb & Pollastri, 2016). Nesse contexto, a N-acetilcisteína (NAC) tem sido utilizada há mais de 30 anos como mucolítico em doença pulmonar obstrutiva crônica (DPOC) e como tratamento para overdose por paracetamol. Nos últimos anos, porém, surgiram evidências que sugerem que a NAC pode ter diversos benefícios terapêuticos em transtornos neuropsiquiátricos (Dean, Giorlando & Berk, 2011; Berk et al., 2013)

A NAC é o derivado N-acetil do aminoácido L-cisteína e é rapidamente absorvido por via oral (Berk et al., 2013). No sistema nervoso central (SNC), L-cisteína é rapidamente oxidada formando cistina. Cistina é o substrato do antiporter cistina-glutamato, o qual transporta o glutamato para o meio extracelular em troca de cistina, regulando dessa maneira os níveis de glutamato extracelular e facilitando, assim, a entrada de cisteína no interior da célula que é o componente limitante da síntese da molécula endógena antioxidante glutationa (GSH) (Figura 1) (Kau et al., 2008; Lushchak & Lushchak, 2012). Por aumentar os níveis de glutationa (GSH), NAC tem ação antioxidante, além disso, possui propriedades anti-inflamatórias por reduzir os níveis de citocinas pró-inflamatórias, incluindo interleucina 6 (IL-6), interleucina 1β (IL-1β) e fator de necrose tumoral alfa (TNF-α) (Dean, Giorlando & Berk, 2011). NAC é um modulador glutamatérgico, regulando a troca neuronal de glutamato através do antiporter cistina-glutamato, além de regular a transmissão dopaminérgica (Dean, Giorlando & Berk, 2011; Berk et al., 2013). NAC também reduz os marcadores de dano oxidativo (Martínez-Banaclocha, 2012; Alboni et al., 2013; Katz et al., 2015; Nouraei et al., 2016; Coles et al., 2017), aumenta o número de sinapses cerebrais (Samuni et al., 2013) e ativa o complexo I mitocondrial (Samuni et al., 2013). Embora haja pesquisas limitadas sobre NAC na doença de Parkinson, alguns dados demonstraram que a NAC é um potencial terapêutico para prevenção e tratamento de DP (Martínez-Banaclocha, 2012; Katz et al., 2015; Nouraei et al., 2016; Coles et al., 2017).

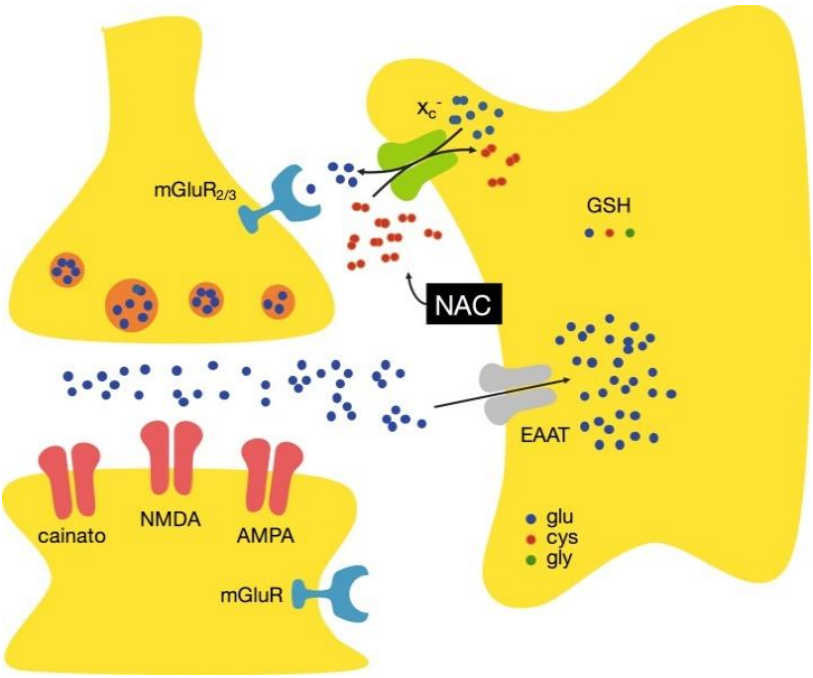


**Figura 1.** Mecanismo de ação da N-acetilcisteína (Adaptado de Berk et al., 2013).

Aspectos não motores da DP incluem depressão, ansiedade, déficit cognitivo e distúrbios de sono. Esses sintomas não-motores respondem muito pouco ou nada às terapias disponíveis atualmente para tratamento de DP (Kalia & Lang, 2015). Nesse sentido, alguns estudos apontam o potencial de NAC como ansiolítico (Egashira et al., 2012; Mocelin et al., 2015; Santos et al., 2017) e antidepressivo (Berk et al., 2011; Magalhães et al., 2011; Costa-Campos et al., 2013; Pilz et al., 2015), o que torna este fármaco uma grande vantagem quando se considera a comorbidade existente entre DP e transtornos psiquiátricos.

### 1.3. Peixe-Zebra

O peixe-zebra (*Danio rerio*), conhecido mundialmente como zebrafish, é um pequeno teleósteo tropical de água doce e tem se tornado cada vez mais utilizado no âmbito da pesquisa científica por apresentar diversas vantagens como animal modelo para o estudo de diversas doenças. Quando comparado com outros modelos animais, como roedores, por exemplo, o peixe-zebra apresenta uma série de vantagens quanto a custo, rápido desenvolvimento e manutenção. Nos primeiros estágios de vida, o

embrião de peixe-zebra é translúcido, possibilitando uma série de técnicas e análises morfológicas nesse organismo *in vivo*. Além disso, estudos envolvendo diversos repertórios comportamentais na área da neuropsiquiatria (Fontana et al., 2018) estão em crescente aperfeiçoamento e padronização, tornando esse organismo modelo uma alternativa para o desenvolvimento de novos fármacos. O peixe-zebra possui 70% de homologia genética com seres-humanos (MacRae & Peterson, 2015), o que permite a extrapolação de resultados obtidos em relação a humanos de maneira mais direta do que aqueles obtidos em invertebrados e isso tem contribuído para investimentos significativos nessa espécie em diversas áreas da pesquisa biomédica translacional.

O conhecimento neuroanatômico e histológico disponível corrobora as sugestões de que o peixe-zebra representa um organismo potencial nos estudos de inúmeras patologias, embora ainda exista a necessidade de se estabelecer paradigmas comportamentais adequados para sua avaliação e comparação com aqueles estabelecidos para outras espécies (Guo, 2004; Bencan, Sledge & Levin, 2009; Egan et al., 2009; Rosemberg et al., 2011; Gebauer et al., 2011). Nos últimos anos, o peixe-zebra tornou-se uma ferramenta poderosa para investigar e desenvolver novos compostos em pesquisas neurológicas e neuropsiquiátricas (MacRae & Peterson, 2015; Fontana et al., 2018), tais como fármacos para o tratamento da doença de Parkinson.

Extensos estudos demonstraram semelhanças entre o sistema nervoso central de mamíferos e as principais regiões encefálicas do peixe-zebra, que se apresentam conservadas em comparação com seus homólogos humanos. Certas áreas do telencéfalo do peixe-zebra são homólogas a regiões dos núcleos da base envolvidos com função motora em mamíferos (Blandini & Armentero, 2012). Neurônios positivos para tirosina hidroxilase (TH) localizados no diencéfalo ventral do peixe-zebra são homólogos à substância negra do mesencéfalo de mamíferos e neurônios da área tegmental ventral e se projetam para o prosencéfalo, possuindo semelhança com o estriado de mamíferos (Parker et al., 2013).

Além dos modelos baseados em manipulação genética, uma série de estudos utilizaram neurotoxinas para modelar DP em peixes-zebra (Feng et al., 2014; Nellore & P, 2015; Wang et al., 2017; Zhang et al., 2017). Uma notável vantagem do modelo de DP em peixe-zebra é que sua barreira hematoencefálica no estádio larval é mais

permeável a neurotoxinas como MPTP e 6-OHDA em comparação com roedores (Feng et al., 2014; Cronin & Grealy, 2017).

Diversos biomarcadores relacionados à DP podem ser estudados no peixe-zebra. Proteínas homólogas às que se encontram alteradas na DP como parkina, pink 1, DJ 1 e LRRK2 são detectadas em diversos estudos (Blandini & Armentero, 2012; Feng et al., 2014). Anichtchik et al., 2004 demonstraram que uma única injeção de 6-OHDA causou diminuição de catecolaminas cerebrais como dopamina e noradrenalina e produziu marcadas alterações comportamentais nesse organismo. Resultados em larvas de peixes-zebra mostraram déficit motor e aumento citocinas pró-inflamatórias TNF-α e cd-11b após exposição a 6-OHDA (Feng et al., 2014). Em um estudo semelhante, Zhang et al. (2017) demonstraram diminuição da TH e déficit motor em larvas de peixes-zebra após exposição a 6-OHDA.

### 1.4. Modelo de Doença de Parkinson induzido por 6-hidroxidopamina

Apenas 5% dos casos de DP são atribuídos a mutações genéticas relacionadas à forma familiar da doença, enquanto que os outros 95% correspondem à forma esporádica (Feng et al., 2014). A forma esporádica é considerada multifatorial, resultando da susceptibilidade a fatores ambientais, genéticos e comportamentais. Nesse contexto, modelos animais de DP induzida por neurotoxinas possuem uma maior abrangência da realidade vista na clínica.

A 6-OHDA é um análogo estrutural da dopamina com alta afinidade por transportadores de dopamina (DAT) que transportam a toxina para dentro dos terminais axonais dos neurônios dopaminérgicos. O mecanismo de ação da 6-OHDA está associado às suas propriedades pró-oxidantes. Uma vez no neurônio, a 6-OHDA se acumula no citosol dos terminais nervosos e sofre auto-oxidação, promovendo formação de peróxido de hidrogênio (Mercanti, Bazzu & Giusti, 2012). Como um mecanismo adicional, a 6-OHDA pode se acumular na mitocôndria, onde inibe o complexo I mitocondrial o que resulta em uma maior produção de espécies reativas de oxigênio (EROs) e um prejuízo na formação do trifosfato de adenosina (ATP), levando à morte dos neurônios por apoptose (Mercanti, Bazzu & Giusti, 2012). Sendo assim, a

lesão obtida com essa neurotoxina representa uma estratégia para modelar a patofisiologia da DP, tornando-se uma ferramenta interessante para investigar os efeitos neuroprotetores de agentes farmacológicos.

## 2. OBJETIVOS

### 2.1. Objetivo Geral:

O objetivo desse estudo foi avaliar os efeitos da N-acetilcisteína (NAC) em um modelo de doença de Parkinson induzido por 6-Hidroxidopamina (6-OHDA) em larvas de peixes-zebra.

### 2.2. Objetivos Específicos

**a.** Padronizar o modelo de DP induzido por 6-OHDA em larvas de peixes-zebra;

**b.** Verificar os efeitos da NAC (1,0 mg/L) no modelo de DP sobre atividade locomotora;

**c.** Verificar os efeitos da NAC (1,0 mg/L) no modelo de DP sobre o comportamento de resposta optomotor;

**d.** Verificar os efeitos de NAC (1,0 mg/L) no modelo de DP sobre padrões morfológicos.

## 3. ARTIGO CIENTÍFICO

**N-acetylcysteine protects against motor, cognitive and morphological deficits induced by 6-OHDA in zebrafish larvae**

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**Keywords**

Parkinson's disease, N-acetylcysteine, 6-hydroxydopamine, zebrafish

**Abstract**

**Background:** Parkinson's Disease (PD) is the second most common neurodegenerative disorder. In addition to its highly debilitating motor symptoms, non-motor symptoms may precede by many years their motor counterparts, which may characterize a prodromal phase of PD. A potential pharmacological strategy is to introduce neuroprotective agents at an earlier stage in order to prevent further neuronal death. N-acetylcysteine (NAC) is an antioxidant and glutamatergic modulator that has shown various therapeutic benefits in other brain disorders but has not been tested in PD models yet.

**Methods:** In this study, we evaluated the potential of NAC to prevent the damage induced by 6-OHDA on motor, cognitive and morphological parameters in a PD model in larval zebrafish.

**Results:** NAC was able to prevent the motor deficits, cognitive injury and morphological alterations caused by exposure to 6-OHDA, which reinforce and broaden the relevance of its neuroprotective effects.

**Discussion:** NAC acts in different targets relevant to PD pathophysiology. Further studies and clinical trials are needed to assess this agent as a candidate for prevention and adjunctive treatment of PD.

## 1. Introduction

Parkinson's Disease (PD) is the second most common neurodegenerative disorder in the world, affecting on average 2-3% of the individuals older than 65 years (Poewe et al., 2017). This condition originates from progressive death of dopamine (DA) neurons in the substantia nigra *pars compacta* of the midbrain and is characterized by motor and non-motor symptoms (Kalia & Lang, 2015). Although the etiology of this multifactorial disease remains unknown, studies demonstrate a key role of oxidative stress in the development of PD in addition to mitochondrial dysfunction, neuroinflammation, dysfunction of the ubiquitin-proteasome system and Lewy body formation (Ryan et al., 2015; Blesa et al., 2015). The main motor symptoms are resting tremor, bradykinesia, muscular rigidity and difficulty in starting or finishing movements, while the equally debilitating non-motor symptoms include mood alterations and cognitive deterioration (Johnson, 2015). Despite being known as a movement disorder, non-motor symptoms have drawn attention because they may precede by many years the motor symptoms, which may characterize a prodromal phase of PD (Pellicano et al., 2007; Berg et al., 2012).

The available drugs for the treatment of PD, such as L-DOPA, are focused on increasing dopaminergic neurotransmission. However, besides inducing several adverse effects and lacking efficacy in the treatment of non-motor symptoms, none of these drugs are able to cure the disease or even slow the progression of neuronal loss (Poewe et al., 2017). A possible explanation for the relative inefficacy of such treatments is related to the tardiness of their onset, which usually begins upon the appearance of motor symptoms, when neuronal death is already at an advanced stage (Berg et al., 2012). Therefore, a potential pharmacological strategy would be to identify individuals in the prodromal phase and to introduce neuroprotective agents at an earlier stage in order to prevent further neuronal death (Dexter & Jenner, 2013).

*Drug repurposing* is a term used to describe the repositioning of known compounds, which are already marketed, to target novel therapeutic purposes. It is an attractive strategy for drug development due to the savings in research, funding and time (Insel et al., 2013; Langedijk et al., 2015; Klug, Gelb & Pollastri, 2016). In this context, N-acetylcysteine (NAC) may be a potential candidate for drug repurposing. It has been used mainly as a mucolytic in chronic obstructive pulmonary disease (COPD) and as a

treatment for paracetamol overdose. More recently, several studies have evidenced that NAC has a multifaceted mechanism of action, presenting antioxidant, anti-inflammatory and neurotrophic effects, besides being a glutamatergic modulator (Berk et al., 2013). Its therapeutic benefits have been demonstrated in clinical trials for several neuropsychiatric conditions (Dean, Giorlando & Berk, 2011).

In recent years, the zebrafish has become a powerful tool to investigate and develop new drugs in neurological and neuropsychiatric research (MacRae & Peterson, 2015a; Fontana et al., 2018). Despite its reduced size and complexity, zebrafish brains have neuroanatomical areas homologous to mammals, including the striatum, therefore, it has also been used and standardized in PD studies as a model for drug screening and investigation of pathophysiology (Rink & Wullimann, 2004; Xi, Noble & Ekker, 2011). In addition to the models based on genetic manipulation, several studies have used neurotoxins to model PD in zebrafish (Feng et al., 2014; Zhang et al., 2017). A remarkable advantage of the zebrafish PD model is that the blood-brain barrier in the larval stage is more permeable to neurotoxins such as 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) as compared to rodents (Feng et al., 2014) (Jackson-Lewis, Blesa & Przedborski, 2012).

Considering the role of accumulated oxidative damage and neuroinflammation on PD pathophysiology, and the multifaceted mechanism of action of NAC, we hypothesized that NAC may be a strong candidate for the prevention of PD. Therefore, the aim of this study was to evaluate the potential of NAC to prevent the injury caused by 6-OHDA exposure on motor and morphological parameters in larval zebrafish.

## 2. Materials and methods

### 2.1. Chemicals and reagents

N-acetylcysteine (NAC), 6-hydroxydopamine hydrobromide 95% (6-OHDA), methyl cellulose and ethyl 3-aminobenzoate methanesulfonate (MS-222) were purchased from Sigma-Aldrich (St Louis, Missouri, USA).

2.2. Animals

Embryos and larvae (0 and 7 days post-fertilization) of AB strain zebrafish (*Danio rerio)* were used. The animals were obtained from our breeding colony, which was maintained in recirculating systems (Zebtec, Tecniplast, Italy) with reverse osmosis filtered water equilibrated to reach the species standard parameters including temperature (28 °C ± 2 °C), pH (7 ± 0.5), conductivity and ammonia, nitrite, nitrate and chloride levels. Water used in the experiments was obtained from a reverse osmosis apparatus (18 MOhm/cm) and was reconstituted with marine salt (Crystal Sea™, Marinemix, Baltimore, USA) at 0.4 ppt. The total organic carbon concentration was 0.33 mg/L. The total alkalinity (as carbonate ion) was 0.030 mEq/L. The animals were kept with a light/dark cycle of 14/10 h. Larvae from the developmental stages used in this study rely on the yolk sac for nutrition and feeding the animals is not necessary. At the end of the experiment, the larvae were euthanized by hypothermia. All protocols were approved by the Animal Care Committee of Pontifícia Universidade Católica do Rio Grande do Sul (#7994/17).

2.3. Experimental design

The experiments were performed according to Figure 1. In a breeding tank, females and males (1:2) were separated overnight by a transparent barrier, which was removed after the lights went on in the following morning. The fertilized eggs that were retained in the bottom of the fitted tank were collected, washed and gently placed in a 6-well plate (15 animals per well) or 24-well plate (4 animals per well), according to the treatment groups and experiment (6-well plate for behavioral tests and 24-weel plate for morphological analysis). A set of animals (n= 8-11) was used for the behavioral tests and another set was used for the morphological analyses (n= 11-12).

At 4 hours post-fertilization (hpf) (Beekhuijzen et al., 2015) the embryos were exposed to 1 mg/L NAC or system water. The medium was not changed until 3 dpf. NAC concentration was determined in a pilot study as well as in our previous studies (Mocelin et al., 2015, 2017).

At 3 days post-fertilization (dpf), 4 dpf, 5 dpf and 6 dpf the animal medium was changed for 250 μM 6-OHDA solution or system water (Zhang et al., 2017). The NAC and 6-OHDA solutions were diluted in system water in a light-protected environment

immediately before use. To avoid interference in the solution pH, we did not use ascorbic acid as a 6-OHDA conservator. For this reason, we opted to replace 6-OHDA solution daily.

At 7 dpf, the motor and morphological parameters of the larvae were analyzed. Embryos and larvae had their mortality and morphology observed daily under the stereomicroscope. There was no difference in the mortality rate between the experimental groups. When applicable, dead animals and the corium were removed. All data were confirmed in duplicate.

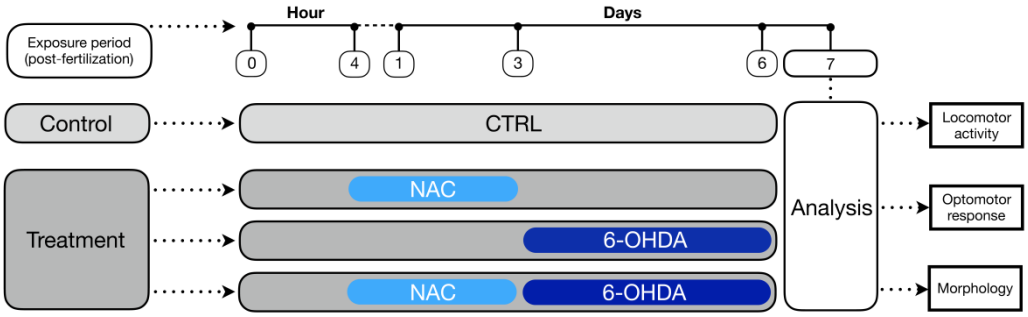


**Figure 1.** Timeline representing the experimental design and treatment groups

2.4. Locomotor behavior

At 7 dpf the larvae were transfered to the experimental room within the animal facility and individually placed in a 24-well plate filled with 2 mL of system water. The locomotor activity was recorded and analyzed during 5 min, following 1 min of acclimation, using Noldus Ethovision XT system (Wageningen, Netherlands). Total distance, mean speed, maximum acceleration, absolute turn angle and immobility time were considered the main parameters of locomotor activity (Altenhofen et al., 2017; Nery et al., 2017). The experiments were performed in a temperature-controlled room (27 ± 2 °C) between 9:00 and 14:00 h.

2.5. Optomotor Response

The optomotor response test allows the assessment of a natural cognitive response behavior, described as the swimming of zebrafish larvae in the same direction of a moving pattern of stripes (Fleisch & Neuhauss, 2006). We performed this test adapted from Creton (2009). The apparatus consists of a Petri dish positioned over a LCD screen.

After being tested for locomotor behavior, the larvae were placed in groups of 10 on the Petri dish filled with 5 ml of system water. After 2 min of acclimation in which the screen was white, the larvae were exposed to a visual stimulus consisting of a moving pattern of red and white stripes (24.5 cm wide and 1.5 cm high). First, the stripes move up at 1 cm/s for 1 min, and then move down at 1 cm/s for 1 min. This pattern repeated 4 times, with a 5 s interval between each 1 min show, in which the screen went white. The entire experiment was recorded and then analyzed by investigators who were blind to the experimental groups. For the data analysis, the Petri dish was virtually divided into two halves (upper and lower) and the number of animals in the stimulus zone (the region towards which the pattern moved) was counted during the 5 s interval of white screen.

### 2.6. Morphological analysis

At 7 dpf the larvae were individually placed in a Petri plate containing 200 µL of 3% methylcellulose with 0.1 g/L ethyl 3-aminobenzoate methanesulfonate (MS-222) solution and each larva was photographed using an inverted stereomicroscope (Nikon, Melville, USA) connected to NIS-Elements Viewer software. Total length, head length, forebrain width, midbrain width and eyes distance were considered the main morphological parameters (Altenhofen et al., 2017) and measured by investigators who were blind to the experimental groups using Image J software. The experiments were performed in a temperature-controlled room (27 ± 2 °C) between 13:00 and 15:00 h.

### 2.7. Statistical analysis

Data were analyzed after normality and homogeneity of variance (D'Agostino-Person and Levene tests, respectively) confirmation using two-way ANOVA to identify the main motor and morphological effects of pretreatment (NAC exposure or not) and treatment (6-OHDA exposure or not) and their interaction, followed by Bonferroni post hoc test. Data were expressed as the mean ± S.E.M. For all comparisons, the significance level was set at $p<0.05$.

## 3. Results

Figure 2 shows the effect of NAC (1 mg/L) on locomotor behavior in 7 dpf larvae exposed to 6-OHDA (250 µM). 6-OHDA caused a decrease in total distance (Fig. 2A), mean speed (Fig. 2B) and maximum acceleration (Fig. 2C), while it increased absolute turn angle (Fig. 2D) and immobility time (Fig. 2E). In all locomotor parameters, NAC was able to prevent the locomotor deficits induced by 6-OHDA. NAC *per se* did not present statistical differences when compared to the control group. Table 1 summarizes the two-way ANOVA analysis.

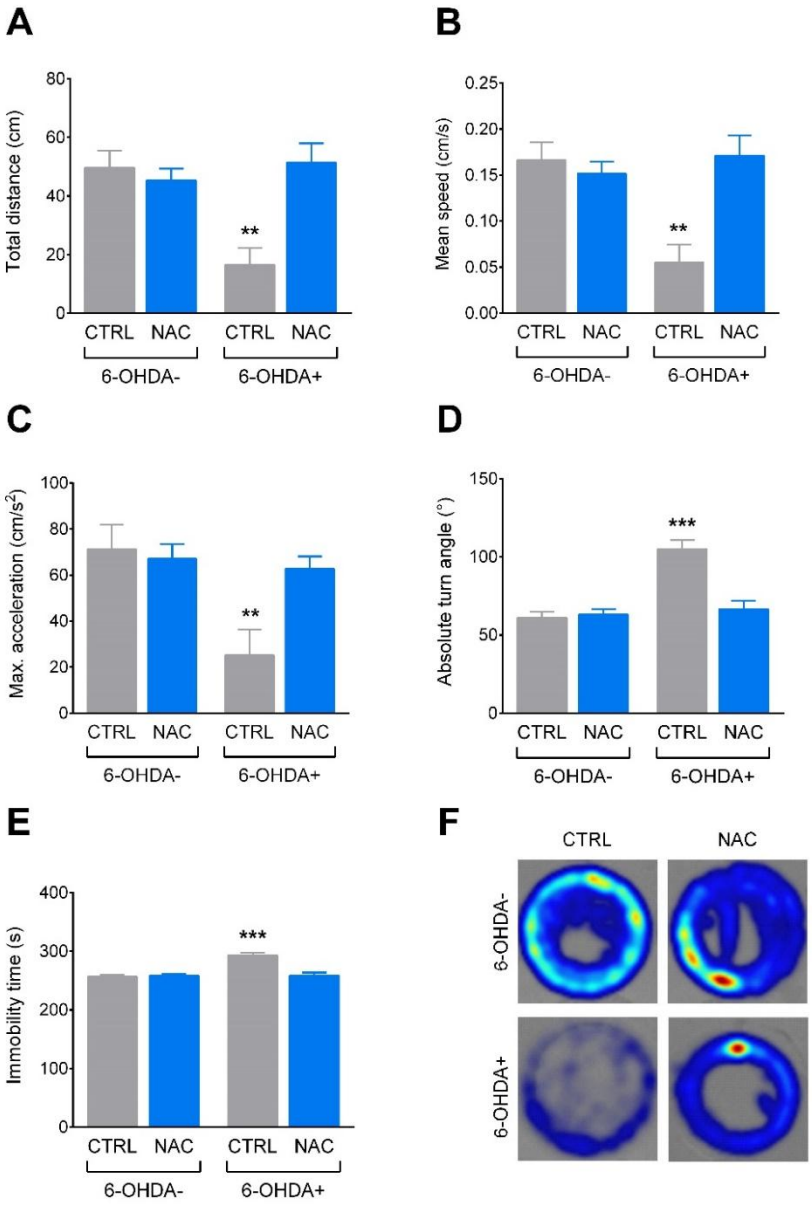

**Figure 2. Effects of NAC on 6-OHDA-induced locomotor behavior deficits in zebrafish larvae.** (A) Total distance travelled, (B) Mean speed, (C) Maximum acceleration, (D) Absolute turn angle, (E) Immobility time and (F) Representative color heatmap of the behavior of one larvae from each treatment group during the trial 5 min duration. Data are expressed as mean ± standard error of mean (S.E.M.). n= 8-11. Two-way ANOVA followed by Bonferroni post hoc test. CTRL, control; NAC, N-acetylcysteine; 6-OHDA, 6-hydroxydopamine. **p<0.01, ***p<0.001 vs. control group (6-OHDA-).

Figure 3 shows the effect of NAC (1 mg/L) on the optomotor response test in 7 dpf larvae exposed to 6-OHDA. Larvae exposed to 6-OHDA spent less time in the stimulus zone, and NAC was able to prevent this optomotor deficit. NAC *per se* did not alter this parameter.

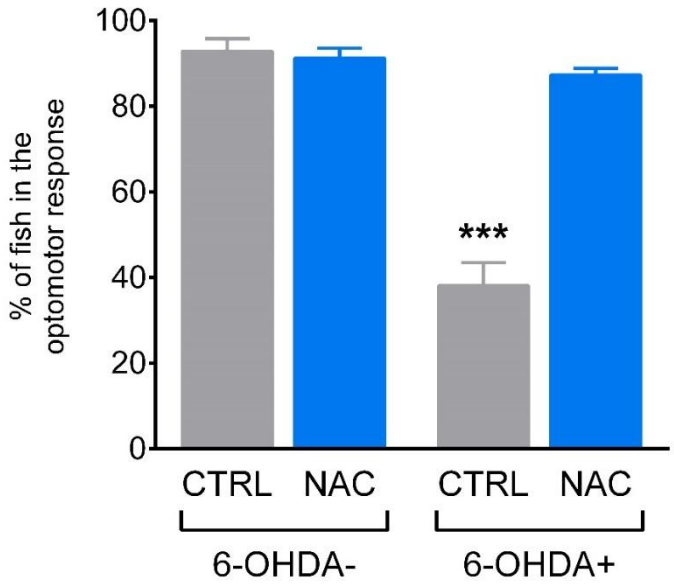


**Figure 3. Effects of NAC on 6-OHDA-induced optomotor response deficit in zebrafish larvae.** Data are expressed as mean ± standard error of mean (S.E.M.). n=10. Two-way ANOVA followed by Bonferroni post hoc test. CTRL, control; NAC, N-acetylcysteine; 6-OHDA, 6-hydroxydopamine. ***p<0.001 vs. control group (6-OHDA-).

Figure 4 shows the effect of NAC (1 mg/L) on morphological parameters in 7 dpf larvae exposed to 6-OHDA (250 µM). 6-OHDA decreased the total length (Fig. 4A) and head length (Fig. 4B), whereas treatment with NAC prevented this effect. NAC *per se* did

not induce morphological alterations. There was no statistical difference in any experimental groups regarding forebrain width (Fig. 4C), midbrain width (Fig. 4D) and eyes distance (Fig. 4E). Table 2 summarizes the two-way ANOVA analysis.

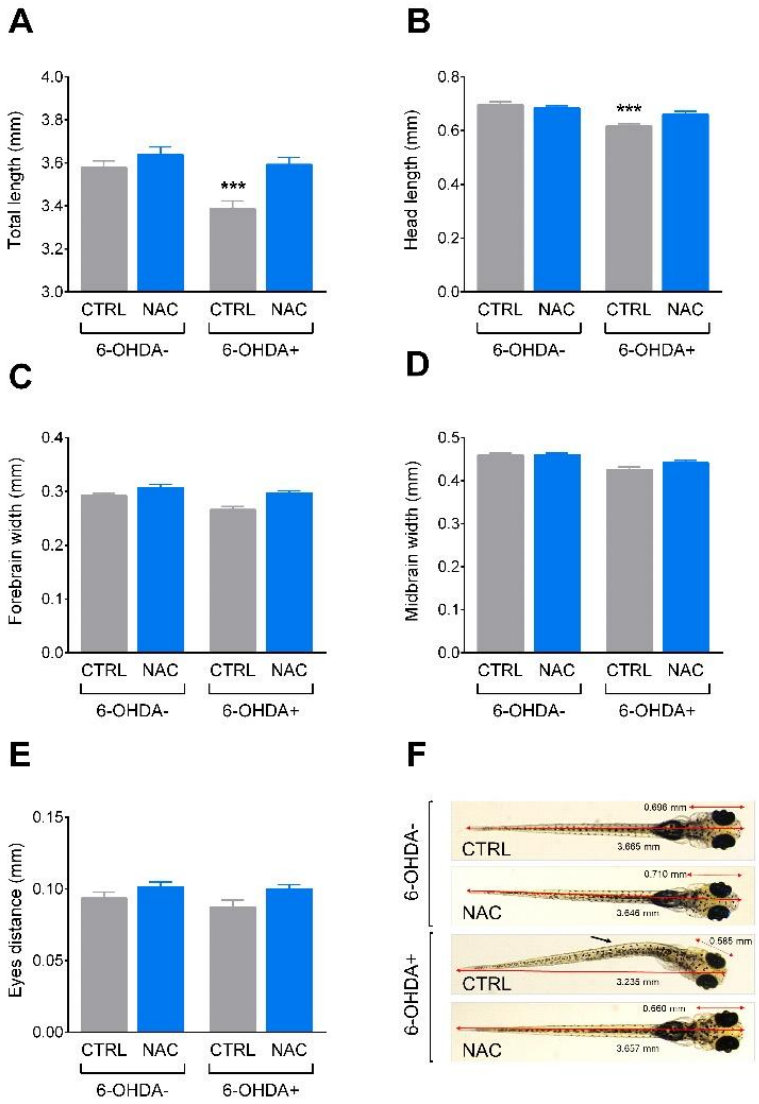


**Figure 4. Effects of NAC on 6-OHDA-induced morphological alterations in larvae zebrafish.** (A) Total length, (B) Head length, (C) Forebrain width, (D) Midbrain width, (E) Eyes distance and (F) Pictures of a larva from each treatment group. The arrow indicates a notable morphological alteration in the shape of the larvae. Data are expressed as mean ± standard error of mean (S.E.M.). n=11-12. Two-way ANOVA followed by Bonferroni post hoc test. CTRL (control); NAC (N-acetylcysteine, 1 mg/L); 6-OHDA (6-hydroxydopamine, 250 µM). ***p<0.001 vs. control group (6-OHDA-).

**Table 1.** The main effects of behavioral analysis and the interaction between pretreatment with NAC and treatment with 6-OHDA.

| Dependent variable | Effects | F-value | DF | P-value |
|---|---|---|---|---|
| Total distance | Interaction | 12.10 | 1,35 | **0.0014** |
| | 6-OHDA | 5.81 | 1,35 | **0.0212** |
| | NAC | 7.31 | 1,35 | **0.0105** |
| Mean speed | Interaction | 12.13 | 1,35 | **0.0014** |
| | 6-OHDA | 5.88 | 1,35 | **0.0206** |
| | NAC | 7.29 | 1,35 | **0.0106** |
| Maximum acceleration | Interaction | 6.46 | 1,35 | **0.0156** |
| | 6-OHDA | 9.36 | 1,35 | **0.0042** |
| | NAC | 4.09 | 1,35 | 0.0506 |
| Absolute turn angle | Interaction | 9.51 | 1,35 | **0.0040** |
| | 6-OHDA | 16.31 | 1,35 | **0.0003** |
| | NAC | 7.33 | 1,35 | **0.0104** |
| Immobility time | Interaction | 17.62 | 1,35 | **0.0002** |
| | 6-OHDA | 19.06 | 1,35 | **0.0001** |
| | NAC | 14.94 | 1,35 | **0.0005** |
| Optomotor response | Interaction | 55.92 | 1,35 | **0.0001** |
| | 6-OHDA | 74.76 | 1,35 | **0.0001** |
| | NAC | 49.15 | 1,35 | **0.0001** |

DF = degrees of freedom. Significant effects ($p<0.05$) are given in bold font.

**Table 2.** The main effects of morphological analysis and the interaction between pretreatment with NAC and treatment with 6-OHDA.

| Dependent variable | Effects | F-value | DF | P-value |
|---|---|---|---|---|
| Total length | Interaction | 4.42 | 1,41 | **0.0417** |
| | 6-OHDA | 11.77 | 1,41 | **0.0014** |
| | NAC | 14.72 | 1,41 | **0.0004** |
| Head length | Interaction | 8.56 | 1,41 | **0.0056** |
| | 6-OHDA | 27.28 | 1,41 | **0.0001** |
| | NAC | 2.87 | 1,41 | 0.0974 |
| Forebrain width | Interaction | 2.40 | 1,41 | 0.1284 |
| | 6-OHDA | 11.87 | 1,41 | **0.0013** |
| | NAC | 18.66 | 1,41 | **0.0001** |
| Midbrain width | Interaction | 2.07 | 1,41 | 0.1578 |
| | 6-OHDA | 24.72 | 1,41 | **0.0001** |
| | NAC | 2.36 | 1,41 | 0.1318 |
| Eyes distance | Interaction | 0.35 | 1,41 | 0.5534 |
| | 6-OHDA | 0.96 | 1,41 | 0.3306 |
| | NAC | 6.58 | 1,41 | 0.0140 |

DF = degrees of freedom. Significant effects ($p<0.05$) are given in bold font.

## 4. Discussion

Our results demonstrated that 6-OHDA at 250 µM is able to induce motor and cognitive deficits and morphological alterations in zebrafish larvae at 7 dpf. Interestingly, NAC (1 mg/L) prevented these effects when administered at the very onset of zebrafish embryos development (4 hpf), showing a clear neuroprotective effect against the neurotoxin.

In the field of animal models of PD induced by neurotoxins such 6-OHDA, the use of zebrafish has increased, probably due to its various benefits when compared to mammal models (Babin, Goizet & Raldúa, 2014; MacRae & Peterson, 2015b). 6-OHDA has been widely used in animal models to mimic pathogenic events and behavioral features observed in PD (Anichtchik et al., 2004; Blandini & Armentero, 2012; Feng et al., 2014; Zhang et al., 2017). This neurotoxin is a reactive structural analogue of DA, uptaken into the neuron by the DA transporter once it crosses the blood-brain barrier. 6-OHDA inhibits the mitochondrial complex I, resulting in an increase of reactive oxygen species (ROS) production and impairment of the ATP generation, which leads to dopaminergic neuron death (Jackson-Lewis, Blesa & Przedborski, 2012).

By causing the death of dopaminergic neurons from important pathways associated with movement regulation, it has been shown in several studies that 6-OHDA is able to cause locomotor deficits in zebrafish larvae (Feng et al., 2014; Zhang et al., 2017) and adults (Anichtchik et al., 2004). According to what is shown in the literature, our results demonstrate that 6-OHDA caused locomotor deficit in all analyzed parameters, causing decrease in distance, mean speed and maximum acceleration and increase in absolute turn angle and immobility time. Our findings show, for the first time, that pre-treatment with NAC is capable of preventing the locomotor deficits induced by 6-OHDA. In another study, NAC improved the behavioral damages and dopaminergic neurons loss induced by rotenone in an animal model of PD in rats, which is in accordance with our results, further suggesting the neuroprotective effects of NAC in behavioral and neurochemical parameters (Rahimmi et al., 2015).

The optomotor response test evaluates zebrafish's cognitive and sensory performance, in addition to its responsiveness to the environment (Maaswinkel & Li, 2003; Creton, 2009). As the dopaminergic system has a key role in cognitive functions and

cognitive impairment is often observed in a more advanced state of Parkinson's disease (Langston, 2006), it is important to evaluate the effects of NAC on cognitive parameters. Thus, our data demonstrated for the first time that larvae exposed to 6-OHDA spent less time in the stimulus zone, presenting a possible cognitive damage. NAC was able to prevent the deficit in optomotor response in larvae exposed to 6-OHDA, demonstrating the neuroprotective role of NAC in pathways related to sensory and cognitive functions of zebrafish larvae.

Studies using tyrosine hydroxylase and dopamine transporter staining showed that after 24 hpf the zebrafish larvae already have functional dopaminergic neurons in areas such as posterior tuberculum in the midbrain and within 4 dpf all dopaminergic pathways are present (Nellore & P, 2015). Our study also shows that 6-OHDA caused morphological changes in zebrafish larvae, producing apparent body curvature and decrease of total length and head length. Thereby, we can assume that the pathways that suffered neuronal death play a key role in regulating the development of the zebrafish larvae. Similar morphological observations were seen in zebrafish larvae exposed to pesticides such as paraquat and rotenone (Bretaud, Lee & Guo, 2004). In the treatment groups that received NAC, no morphological changes were observed, even when exposed to 6-OHDA. Because of its mechanism of action, NAC is likely to have protected these pathways, preventing the morphological changes caused by 6-OHDA. NAC (100 mg/kg) was able to increase the levels of the dopaminergic marker tyrosine hydroxylase (TH) in the striatum of mice exposed to 6-OHDA, reinforcing the important effect of NAC in the preservation of the dopaminergic system (Nouraei et al., 2016).

NAC is an antioxidant precursor of glutathione (gamma-glutamylcysteinylglycine; GSH), displaying anti-inflammatory properties by reducing pro-inflammatory cytokine levels, including interleukin (IL)-6, IL-1β and tumor necrosis factor alpha (TNF–α) (Dean, Giorlando & Berk, 2011). NAC also shows glutamatergic modulator activities by regulating neuronal exchange of glutamate through the cystine-glutamate antiporter, in addition to regulating the dopaminergic transmission (Dean, Giorlando & Berk, 2011; Berk et al., 2013). NAC also reduces oxidative damage markers (Alboni et al., 2013), increases the number of brain synapses (Samuni et al., 2013) and activates the mitochondrial complex I (Samuni et al., 2013). Although there is limited research about NAC in Parkinson's disease, some studies have demonstrated that NAC has potential as a therapeutic strategy for PD

prevention and treatment (Martínez-Banaclocha, 2012; Katz et al., 2015; Nouraei et al., 2016; Coles et al., 2017).

Studies have indicated NAC effects as an anxiolytic (Mocelin et al., 2015; Santos et al., 2017) and antidepressant drug (Magalhães et al., 2011; Berk et al., 2013; Costa-Campos et al., 2013; Pilz et al., 2015). Therefore, NAC has advantages over the existing therapies since, besides having a potential preventive effect, it may be able to treat the non-motor aspects of PD, which include depression, anxiety, cognitive impairment and sleep disturbances (Klockgether, 2004).

It is increasingly necessary to develop drugs with a multifaceted mechanism capable of acting on several targets that are altered in neurodegenerative diseases, such as Parkinson's Disease, more effectively and with fewer adverse effects. Translational research aims to serve as a powerful tool in the development of pharmacological interventions that fulfill this goal (Pickart & Klee, 2014). Our results propose the investigation of the effect of NAC in the prevention and treatment of PD, showing a robust protective effect of NAC in behavioral, morphological and cognitive parameters in a translational model of PD in zebrafish. However, further research is needed to evaluate the action of NAC in other important PD markers. Therefore, we intend to perform additional studies to analyze the role of NAC in markers of oxidative stress, apoptosis and tyrosine hydroxylase in the present model of PD in zebrafish.

In conclusion, this study demonstrated that NAC was able to prevent the behavioral deficits and morphological alterations induced by 6-OHDA, which supports its powerful neuroprotective effect. For having differentiated profile and mechanism of action, NAC is a strong candidate for the prevention and treatment of PD, acting in different aspects of the disease.

## 5. Acknowledgements

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## 4. CONCLUSÃO

Esse estudo demonstrou que a NAC foi capaz de prevenir os déficits comportamentais e as alterações morfológicas induzidas pela neurotoxina 6-OHDA, o que suporta seu efeito neuroprotetor. Por ter perfil e mecanismo de ação diferenciados, a NAC é uma candidata para a prevenção e tratamento da DP, atuando em diferentes aspectos da doença.

## 5. PERSPECTIVAS

Com o objetivo de complementar os dados obtidos nesse estudo, assim como dar continuidade na investigação do papel de NAC na prevenção de DP, serão realizados mais experimentos a fim de elucidar o mecanismo pelo qual esse fármaco produz os efeitos observados. Entre as técnicas que serão empregadas em estudos futuros estão:

**i. Laranja de acridina:** Será realizada a técnica de coloração de laranja de acridina com o objetivo de analisar o efeito anti-apoptótico de NAC no modelo de DP induzida por 6-OHDA em larvas de peixes-zebra.

**ii. 2',7'-dichlorodiidrofluoresceina diacetato (H2DCFDA):** Será realizada a técnica de 2',7'-dichlorodiidrofluoresceina diacetato para analisar a atividade antioxidante de NAC no modelo de DP induzida por 6-OHDA em larvas de peixes-zebra.

**iii. Quantificação de Superóxido dismutase (SOD), Catalase (CAT) e Glutationa (GSH):** Serão realizadas as técnicas de quantificação de SOD, CAT e GSH com o objetivo de analisar o papel de NAC no modelo de DP induzida por 6-OHDA em larvas de peixes-zebra quanto a parâmetros bioquímicos de defesas antioxidantes.

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## 7. ANEXOS

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

**PRÓ-REITORIA DE PESQUISA**

**Comissão De Ética No Uso De Animais**

CEUA UFRGS

### *CARTA DE APROVAÇÃO*

**Comissão De Ética No Uso De Animais analisou o projeto:**

**Número:** 31896
**Título:** AVALIACAO DOS EFEITOS DA N-ACETILCISTEINA EM MODELO DE DOENCA DE PARKINSON EM PEIXES-ZEBRA

**Vigência:** 01/09/2016 à 01/08/2018

**Pesquisadores:**

**Equipe UFRGS:**

ÂNGELO LUIS STAPASSOLI PIATO - coordenador desde 01/09/2016
RADHARANI BENVENUTTI - Aluno de Mestrado desde 01/09/2016
Ricieri Naue Mocelin - Aluno de Doutorado desde 01/09/2016
Matheus Felipe Marcon - Aluno de Doutorado desde 01/09/2016

***Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 21/11/2016 - SALA 330 DO ANEXO I - PRÉDIO DA REITORIA DA UFRGS/CAMPUS CENTRO/UFRGS, em seus aspectos éticos e metodológicos, para a utilização de 5 machos e 5 fêmeas peixes-zebra (Danio rerio) (progenitores) proveniente do Biotério do Depto. de Bioquímica da UFRGS, e 475 peixes-zebra adultos de ambos os sexos e 540 larvas de peixe-zebra, resultantes do cruzamento dos progenitores no Biotério do Depto de Farmacologia da UFRGS, de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.***

Porto Alegre, Segunda-Feira, 5 de Dezembro de 2016

MARCELO MELLER ALIEVI
Coordenador da comissão de ética

# SIPESQ

**Sistema de Pesquisas da PUCRS**

Código SIPESQ: 7994 Porto Alegre,3 de julho de 2017

Prezado(a) Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou o Projeto de Pesquisa "AVALIAÇÃO DOS EFEITOS DA N-ACETILCISTEÍNA EM MODELO DE DOENÇA DE PARKINSON EM PEIXES-ZEBRA" coordenado por MONICA RYFF MOREIRA VIANNA.

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está autorizada a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Duração do Projeto: 03/07/2017 - 03/01/2019

| Nº de Animais | Espécie |
|---|---|
| 1383 | Danio rerio |
| Total de Animais: 1383 | |

Atenciosamente,

Comissão de Ética no Uso de Animais(CEUA)