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Assessment of toxicity and oxidative stress associated with omeprazole in *Caenorhabditis elegans*

Gabriela Endres da Rocha Pompeo*, Roberta Rodrigues Zorzo, Bianca Bordignon Fraga, Mariele Feiffer Charao and Magda Susana Perassolo

Universidade Feevale, RS-239, 2755, 93525-075, Novo Hamburgo, Rio Grande do Sul, Brazil. *Author for correspondence. E-mail: endresgabriela@gmail.com

ABSTRACT. Omeprazole is an inhibitor of the proton pump which has been extensively studied presently due to the evidence of a potential toxicity related to the generation of reactive species of oxygen (ROS) to its chronic users. Thus, this study aimed to assess the toxicity and oxidative stress of omeprazole in *Caenorhabditis elegans*. Survival assays, nematode body length and ROS generation trials were carried out after the exposure of *C. elegans* to omeprazole. The omeprazole used for the treatment of nematodes was the commercial standard in pellets. The pellets were dissolved in a bicarbonate solution (84 mg mL^{-1}) to a 10 mg mL^{-1} concentration. The N2 wild strain of *C. elegans* was kept in NGM medium, incubated at 20°C and supplemented with *E. coli* OP50 as food source. Nematodes in L1 stage were obtained through synchronization and, afterwards, 1500 nematodes were exposed to 5 concentrations of omeprazole (62.5 µg mL^{-1}, 125 µg mL^{-1}, 250 µg mL^{-1}, 500 µg mL^{-1}, 1 mg mL^{-1}). The generation of ROS was assessed through fluorescence in a 0.05 mM 2',7'-dichlorofluorescein diacetate (DCF-DA) solution. The exposure of *C. elegans* to omeprazole reduced size ($p < 0.05$), survival ($p < 0.05$) and increased generation of ROS ($p < 0.05$) in a dose-dependent manner. The lethal dose (DL50) was determined as 968 µg mL^{-1}. Bicarbonate, omeprazole diluent, was tested as control and did not present any significant alteration ($p > 0.05$). The decrease in survival and body length of nematodes treated with omeprazole and the generation of ROS confirm its toxicity.

Keywords: omeprazole; proton pump inhibitors; toxicity; reactive species of oxygen; *Caenorhabditis elegans*.

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Introduction

Omeprazole was the first drug of the class of proton pump inhibitors (PPI) to arise in Brazil and continues to be the most utilized drug to date (Salgado et al., 2019). It is part of the National List of Essential Medicines (RENAME) of Brazil and the Brazilian Health Regulatory Agency (ANVISA) only allows its use under medical prescription, however its self-medicated use is a reality. Omeprazole is chemically stable and does not present inhibitory activity at neutral pH. At pH 5 and lower it undergoes rearrangement, forming a sulfenic acid and sulfenamide which react with the thiol groups of the enzyme H^+/K^+-ATPase, the proton pump, and therefore irreversibly inhibit it. This inhibition prevents the production of hydrochloric acid by the parietal cells due to the stimulation of cholinergic, histaminergic and gastrinergic receptors (Korolkovas & França 2006).

It is found in the literature that about 15% of an omeprazole dose may cross the blood-brain barrier and reach the central nervous system (CNS) (Ortiz-Guerrero et al., 2018). Frequent use of PPIs has the potential to cause serious health consequences for those who use these medications. Studies have shown that patients who use PPIs have an increased risk of developing neurocognitive diseases (Gomm et al., 2016).

The oxidative stress (OS) is characterized as the unbalance in the production of reactive oxygen species (ROS), known as: superoxide radicals (O_2^-), hydroxyl (OH^-), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2) (Pizzino et al, 2017). The majority of ROS (90%) is produced during the oxidative phosphorylation in the mitochondria. Cytochrome c oxidase, a type of electron transfer protein present in the mitochondria, inhibits the formation of intermediate oxygen species. Nonetheless, a fraction of free radicals evades the cytochrome and for this fraction, the cell depends on the protection of the antioxidant enzyme system (Pinto, 2017).

An antioxidant may be defined as any substance which slows, prevents or removes oxidative damage to a target molecule (Sies, 2020). Antioxidants may be endogens, present inside the cells, or exogenous, which

need to be obtained throughout diet, for instance. The enzymes superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GSR) and catalase are the most important endogen antioxidants in our organism (Pinto, 2017).

Brain tissue presents a high consumption of oxygen and low amount of antioxidants, which may allow it to be susceptible to oxidative damage. A recent study was carried out to assess *in vitro* the behavior of erythrocytes exposed to omeprazole and the consequences on oxidative stress levels. Several oxidative stress parameters demonstrated alterations when erythrocytes were exposed to the treatment. After 48 hours of exposure, there was a significant decrease in superoxide dismutase (SOD) and glutathione peroxidase (GPX) levels, which confirms the generation of oxidative stress due to omeprazole (Naveed et al., 2020).

Several studies have chosen to use *in vivo* models in order to prove theories involving OS. *Caenorhabditis elegans (C. elegans)* is amongst them. *C. elegans* is a nematode which measures about 1mm in length and presents excellent characteristics to be an *in vivo* model. Its systemic anatomy involves: neural system, digestive system, immune system and reproductive system, which grant reliability to the findings regarding this nematode (Wu et al., 2019).

Due to a fully described nervous system containing 302 neurons in hermaphroditic adults (383 in males), divided into 118 morphologically distinct classes and 56 glia cells, which combined form more than 7600 synapses (White et al., 1986), *C. elegans* has been increasingly used in neuroscience. Furthermore, the nematode is the only organism which has its wiring diagram of the brain completely determined (Ruszkiewicz et al., 2018).

Recently, it was demonstrated that damage involving particles at nanometer scales, in *C. elegans*, presents as its main toxic effect damage to oxidative stress levels (Wu et al., 2019).

Its short life cycle allows to demonstrate the chronic effects caused by some toxicants, which is practically infeasible in mammals with a long-life cycle. It is also known that when the same drug is tested in mammals and *C. elegans,* the results obtained are extremely similar (Ruszkiewicz et al., 2018). Therefore, this study aimed to assess in the alternative model *C. elegans* the toxicity and oxidative stress caused by the use of omeprazole.

Material and methods

Omeprazole solution

Omeprazole used for the treatment of nematodes was the commercial standard in pellets with a correction factor of 9.62 mg and at 10%. The pellets were dissolved in a bicarbonate solution (84 mg mL^{-1}) to a concentration of 10 mg mL^{-1}. The following treatment solutions were prepared from the stock solution: 62.5 µg mL^{-1}, 125 µg mL^{-1}, 250 µg mL^{-1}, 500 µg mL^{-1}, 1 mg mL^{-1}.

Caenorhabditis elegans

The N2 wild strain obtained from the *Caenorhabditis* Genetic Center of the University of Minnesota (USA) was used. The strain was kept in NGM (Nematode Growth Medium) supplemented with *Escherichia coli* (OP50) as food source and stored in a Bio-Oxygen Demand (BOD) Incubator at 20°C (Brenner, 1974).

Synchronization

In order to carry out the assays, firstly the strain is synchronized, aiming to obtain nematodes in the same larval stage. Briefly, the nematodes carrying embryos are removed from the plate and transferred to a Falcon tube (50 mL). Afterwards, the bleaching solution (NaOH 0.45 N, HOCl 2%, w v^{-1}) is added to promote the rupture of the nematodes and the release of the eggs. The eggs are removed through a sucrose gradient (30%, m v^{-1}) and arranged on a plate containing NGM. After 12-14 hours, the eggs hatch and the nematodes reach the L1 larval stage.

Treatment

For the treatment, 1500 nematodes in the L1 stage were used. Five different omeprazole concentrations were tested (62.5 µg mL^{-1}, 125 µg mL^{-1}, 250 µg mL^{-1}, 500 µg mL^{-1}, 1 mg mL^{-1}). The nematodes were exposed in liquid medium and kept in a rotary homogenizer for one hour in a BOD incubator. Moreover, treatment with the solution of the omeprazole dissolution (sodium bicarbonate 84 mg mL^{-1}) was performed. The control

group was treated with M9 solution (3 g L^{-1} of KH_2PO_4, 6 g L^{-1} of Na_2HPO_4 and 5 g L^{-1} of NaCl). After the exposure period, nematodes were transferred to Petri dishes containing NGM and supplemented with *E. coli* (OP50) (Benedetto et al., 2010). The assays were carried out in duplicate and repeated three times.

Survival

The survival assay was employed 24 hours after the treatment. The alive nematodes were counted in 10 random quadrants of each dish using a stereomicroscope. The approximate amount of alive nematodes in the entire dish was calculated afterwards. The results obtained were used to develop the survival curve and determine the Lethal Dose 50 (Brenner, 1974). The control group was considered as 100% survival rate.

Body length

When the nematodes reached adulthood, photos of 40 nematodes from each treatment were taken using a stereomicroscope with camera. Subsequently, the length from tail to head was measured through the ImageJ® software - version 1.51k (Rasband et al., 1997; Charão et al., 2015).

Determination of ROS

In order to determine ROS levels, the nematodes were washed and, after the last centrifugation, were resuspended in 100 µL of NaCl 10% and transferred to a 96-Wells plate. Thereafter, 100 µL of the 2',7'-dichlorofluorescin diacetate (DCF-DA) 0.05 mM solution was added. The reading was carried out for 60min in a microplate reader at 488nm and 520nm (excitation and emission, respectively). In the presence of ROS, DCF-DA is oxidized to the fluorescent product dichlorofluorescin (DCF). The results were expressed as percentage of fluorescence intensity in relation to control group.

Statistical analysis

The results were expressed in mean and standard deviation for data of normal distribution and median and interquartile ranges for data with non-normal distribution. Results expressed in % were normalized in relation to control group (100%). Statistical analysis was carried out utilizing the Prisma software version 8.0 (Motulsky, 1989). The Shapiro-Wilk test was performed in order to evaluate the data distribution. For assessing the difference between groups, one-way ANOVA test was performed, followed by Dunnett's test. Analyzes were conducted with a 95% confidence interval, therefore a $p < 0.05$ was considered significant. Survival, body length and ROS generation graphs were generated using the same statistical software.

Results and discussion

Omeprazole-induced reduction in body length

The effect of omeprazole in *C. elegans* was tested using 5 different concentrations in duplicate and 3 repetitions (n = 3). All concentrations tested demonstrated a reduction in body length in relation to the length of the nematode (Figure 1). The concentration exhibiting the most evident effect on the length of *C. elegans* was 1 mg mL^{-1}. The bicarbonate, diluent of omeprazole in the study, was tested as control and did not present any significant alteration regarding the body length of the nematodes.

Survival reduction

The values referring to the *C. elegans* survival count presented significant counting differences at all concentrations (Figure 2). The concentration 1mg mL^{-1} presented the least amount of survivors. Bicarbonate, diluent of omeprazole in the study, was tested as control and did not present any significant alteration regarding the survival of the nematodes.

ROS levels

When determining ROS levels, it was observed that the percentage of fluorescence intensity increased proportionally to the increase in concentrations (Figure 3). The drug was tested in isolation and it is confirmed the fluorescence levels were not affected. At 1 mg mL^{-1} concentration, fluorescence levels were lower than expected. However, it is suspected this outcome is due to the higher death of nematodes at this concentration.

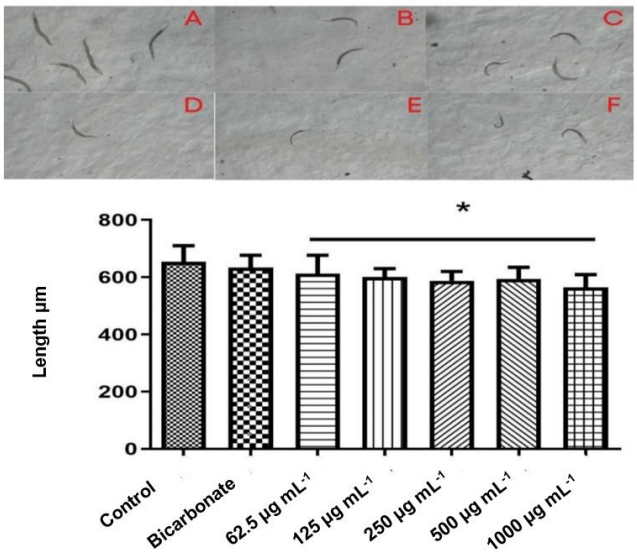

Figure 1. Body length of *C. elegans* after exposure to omeprazole. Graphic representation of the length of nematode. Significant difference was confirmed through the Kruskal-Wallis method, followed by Dunn's post hoc test. The asterisk indicates significant difference when compared to control group. *p < 0.0001. The photos of the nematode represent the decrease of body length. (A) Control treatment; (B) 62.5 µg mL^{-1}; (C) 125 µg mL^{-1}; (D) 250 µg mL^{-1}; (E) 500 µg mL^{-1}; (F) 1 mg mL^{-1}.

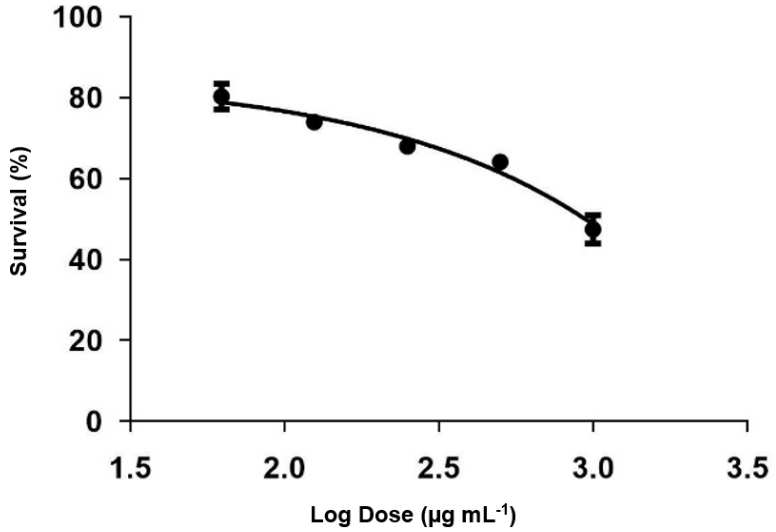

Figure 2. Lethality assay. Significant differences were determined through ANOVA and followed by Dunnet's test. LD50 was determined as 968 µg mL^{-1}.

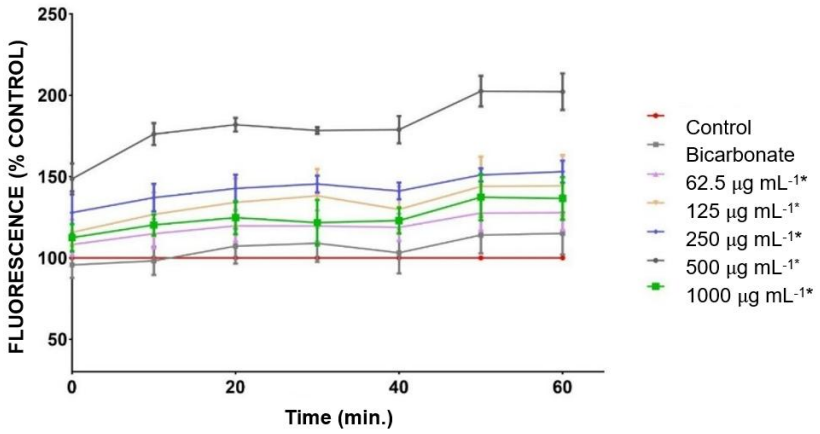

Figure 3. Determination of oxidative stress levels. Percentage representation of the fluorescence levels originating from the oxidation of the fluorescent product dichlorofluorescein (DCF). ANOVA followed by Dunnet's test were employed to confirm significant difference. *p < 0.05.

The control group did not present any significant alteration ($p > 0.05$) when compared to the dilution solution of bicarbonate. Every other concentration demonstrated significant alterations. The concentration exhibiting the greater amount of ROS generation in relation to the control group was 500 μg mL^{-1} ($p < 0.0001$).

This study aimed to associate oxidative stress and toxicity to the chronic use of omeprazole in *C. elegans*. The parameters related to length and survival of the nematode demonstrated coherent results. There was a decrease in survival which was directly related to the increase of concentration. In addition, the body length of nematodes behaved in a similar manner. The length of *C. elegans* decreased whilst the concentrations increased. DCF-DA generated higher fluorescence intensity according to the increase of concentrations, except at 1 mg mL^{-1} in which the generation of DCF remained low.

Caenorhabditis elegans is the only organism which possesses a fully described nervous system and it is considered to be structurally and functionally similar to mammals (Ruszkiewicz et al., 2018). The neurotransmitters utilized by the organism are glutamate (Glu), γ-aminobutyric acid (GABA), dopamine (DA), serotonin (5-hydroxytryptamine; 5-HT) and acetylcholine (ACh), thereby making its CNS become even more mammal-like (Brownlee & Fairweather, 1999). Several studies report the evaluation of the effects of toxins through experiments with *C. elegans* is a corroborated method (Ruszkiewicz et al., 2018). Due to the previously reported data, it is relevant to emphasize *C. elegans* is an important evaluator of damage to the CNS.

In a study carried out at the Rostock University in Germany, data from 73,679 patients, 75 years old or older between the years of 2004 and 2011, were analyzed. The data came from a German health insurance unit entitled 'Allgemeine Ortkrankenkassen (AOK)'. The performed analyses demonstrated a significantly increased risk of developing dementia for individuals using PPI, confirming data from a previous study by the same authors (Gomm et al., 2016).

Regarding the analyses of the reactive oxygen species (ROS), at 62.5 μg mL^{-1}, 125 μg mL^{-1}, 250 μg mL^{-1}, 500 μg mL^{-1} concentrations, the amount of ROS gradually increased according to the concentrations. Thus, it confirms the generation of oxidative stress by omeprazole at these concentrations. At 1 mg mL^{-1} concentration, there was a low percentage of fluorescence. It is believed the effect has been generated due to the high mortality rate of the nematodes at this concentration. Therefore, in the absence of worms there is no DCF generation. In the literature, several studies correlate these oxidative reactions to aging and some pathological processes such as: cardiovascular diseases, pulmonary diseases, diabetes, cancer and neurodegenerative diseases (Gemelli et al., 2013). The brain and nervous system are susceptible to oxidative damage and oxidative stress which, consequently, influence the cause of neurodegenerative diseases (Gemelli et al., 2013).

As previously mentioned, oxidative stress may occur due to an imbalance between the production and removal of reactive oxygen species (ROS). The mitochondria is considered the main source of ROS (Back et al., 2012). In mammals, the main metabolic source which produces energy is the mitochondria, as it is responsible for the formation of ATP in the whole body throughout oxidative phosphorylation. To the present moment, it is indicated that the mitochondrial processes of *C. elegans* are similar to the ones of mammals (Moreno-Arriola et al., 2014).

In another recently published study, alterations resulting from the use of omeprazole in humans were evaluated. 75 patients were selected, 35 were omeprazole users (OU) for at least six months uninterrupted and 30 non users of omeprazole (NUO) for at least one year. The researchers assessed the levels of vitamin B12, oxidative stress and cognitive aspects. There was no significant difference between the groups regarding the levels of vitamin B12. However, oxidative stress parameters demonstrated higher levels of the ferric reducing ability of plasma (FRAP) and lower levels of glutathione peroxidase (GPX) on OU when compared to the NUO group. Regarding cognitive tests, OU group performed worse overall than the NUO group (Dries et al., 2022).

Analyzing the results obtained from the body length, survival and DCF-DA assays, it is possible to observe the same pattern. Therefore, all the parameters evaluated are affected according to the increase of omeprazole concentrations. The three tests evaluating toxicity damage and ROS generation may be correlated to data found in the literature. The current study is the first to assess this class of drugs using the *C. elegans* model.

Conclusion

Our findings, with their limitations regarding an *in vivo* study with the *Caenorhabditis elegans* model, lead us to a possible consideration of proposing the chronic use of omeprazole as toxic. The damage to the body length

and survival of the nematode may be related to the generated oxidative stress, which already presents consistent evidence to be crucial in the degeneration which occurs in the Nervous System (Simonian & Coyle 1996).

Therefore, we may conclude the exposure of *C. elegans* to omeprazole leads to toxicity and increase of oxidative stress. Our findings are coherent to previous studies, validating the animal model employed in the current study. In addition, we suggest further research involving PPI and *C. elegans* is carried out in order to achieve a definitive outcome regarding the proposed theme.

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