UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

DISCIPLINA DE TRABALHO DE CONCLUSÃO DE CURSO DE FARMÁCIA

Piperazine designer drugs elicit neurotoxicity in the alternative *in vivo* model *Caenorhabditis elegans*

Caroline Souto

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Trabalho de conclusão de curso apresentado como pré-requisito para obtenção do Grau de Farmacêutica

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“No fim das contas, podemos aguentar muito mais do que imaginamos.”

- Frida Kahlo

Este Trabalho de Conclusão de Curso foi redigido sob a forma de artigo o qual foi elaborado segundo as normas da revista “Neurotoxicology”, apresentadas em anexo.

Piperazine designer drugs elicit neurotoxicity in the alternative *in vivo* model *Caenorhabditis elegans*

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ABSTRACT

Piperazine designer drugs are a group of synthetic drugs of abuse that have appeared on the illicit market since the second half of the 1990s. The most common derivatives are 1-benzylpiperazine (BZP), 1-(4-methoxyphenyl)piperazine (MeOPP), and 1-(3,4-methylenedioxybenzyl)piperazine (MDBP). Generally, they can be consumed as capsules, tablets, but also in powder or liquid forms. Although less potent than amphetamines, piperazines have dopaminergic and serotonergic activities. The aim of this work was to evaluate the neurotoxic effects of BZP, MeOPP and MDBP using C. elegans as in vivo model through acute toxicity, development, reproduction, and behavior tests. The LD50 for BZP, MeOPP e MDBP was 52.21, 5.72, and 1.22 mM respectively. All concentrations were accompanied by a significant decrease in the body surface of the worms, indicating alteration of the development and decrease in reproduction. Worms exposed to piperazine designer drugs also presented a decrease in locomotor activity and mechanical sensitivity, suggesting the possible dysfunction of the nervous system. Neuronal damage was confirmed though the decrease in fluorescence of BY200 strains, indicating loss of dopaminergic transporters (DATs). In summary, we suggest that piperazine designer drugs leads to neuronal damage, which might be the underlying cause of altered behavior.

Keywords:

Neurotoxicity, *C. elegans*, piperazine designer drugs

1. INTRODUCTION

The worldwide consumption of synthetic drugs of abuse, known as designer drugs, has been raising year after year. They are among the most used drugs by Western civilization (Gahlinger, 2001). Commonly produced in clandestine laboratories, the designer drugs are often synthesized from other chemical substances that have already a well-defined biological activity. Among these effects, increased communicability, empathy and self knowledge are the most described (Costa,2004; Lanaro *et al.*, 2010). The main representative of the category is 3,4-methylenedioxymethamphetamine (MDMA), popularly known as ecstasy. Nevertheless, other substances have also been seized for its recreative purposes, such as the piperazine designer drugs. These compounds represent a wide chemical category of six atoms cyclic structure with two nitrogens in opposite positions (Kovaleva *et al.*, 2008).

The piperazines designer drugs are considered a new group of synthetic drugs of abuse which emerged at the illicit market from the second half of the 1990s (Byrska *et al.*, 2010). Their potential as recreative drugs is highlighted by its psychoactive properties, such as, for instance, states of euphoria that are comparable to the ones obtained consuming amphetamines or ecstasy, and, therefore, are considered a cheap and safe alternative to other drugs (Yeap *et al.*, 2010). They began to be used as substitutes of ecstasy, once they have similar stimulant and hallucinogen effects and identical physical appearance.

The derivatives are divided between two groups: the benzylpiperazines, such as the N-benzylpiperazine (BZP) and the 1-(3,4-methylenedioxybenzyl)piperazine (MDBP); and the phenylpiperazines, such as the 1-(4-methoxyphenyl)piperazine (MeOPP) (Arbo *et al.*, 2012), (Figure 1). Generally, they can be consumed as pills, capsules, but also as powder or in liquid forms (Gee *et al.*, 2005).

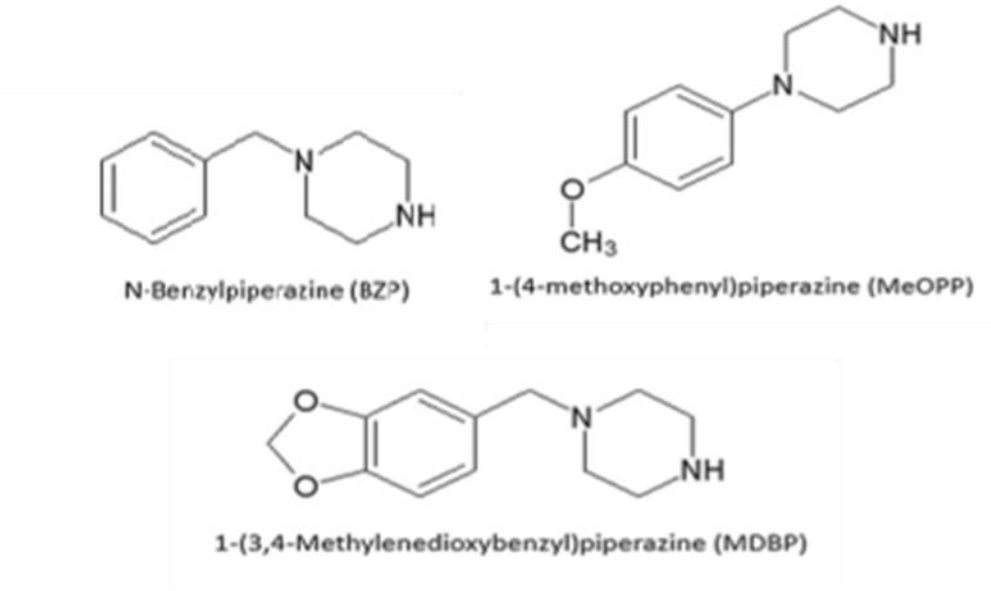


Figure1. Chemical structure of the most common piperazine designer drugs

Although less potent than amphetamines, piperazines have dopaminergic and serotonergic activities. They can act at noradrenergic, dopaminergic and serotonergic synapses causing the same neurotoxic effects as amphetamines or MDMA (Baumann *et al.*, 2005). In vivo studies have confirmed a clear stimulating behavioural pattern associated with the increase of dopamine and liberation of serotonin (Baumann *et al*., 2005; Meririnne *et al.*, 2006; Yarosh *et al.*, 2007). In small doses, the effects tend to be slight, producing sensations of euphoria and vigil. The most common symptoms include insomnia, headaches, nausea, anxiety, depression, paranoia and hearing hallucinations. The ingestion of high doses results in sympathomimetic toxicity, the patients feel palpitations, tachycardia, and hypertension. The neurological effects include shivers, myoclonus and convulsion (Elliott, 2011; Arbo *et al*., 2012; Musselman e Hampton, 2014). In this way, consumers of these drugs present a significant reduction of neuroreceptors and neurotransmitters, confirming its neurotoxic potential in humans (Tsutsumi *et al.*, 2005; Baumann *et al*, 2005).

Toxicological studies are vital to elucidate the toxicity of any substance. In order to minimize the use of complex animals in scientific experiments, in 1959 the concept of the “3 Rs”: *Reduction*, *Refinement*, *Replacement* was proposed. That is reduction in the number of animals in experimentation,

refinement of the procedures (decreasing pain and stress to the animals) and replacement of animal methods to those “non-animals” (*in silico, in vitro*, *ex vivo*), including the use of alternative methods (Boyd *et al.*, 2007). Thereby, in recent years, an alternative and well-characterized model has been of great relevance for toxicological evaluation, the microscopical nematoid *Caenorhabditis elegans* (Gami e Wolkow, 2006; Kaletta, T. e Hengartner, M. O., 2006). This in vivo model is useful due to a strong similarity between *C. elegans* and the mammals in cellular and molecular principles, 60 to 80% of the nematoid genes possesses homologous in human being (Kaletta, Titus e Hengartner, Michael O, 2006; Charão *et al.*, 2015).

Therefore, the aim of this study was to evaluate the acute toxicity of three piperazine designer drugs (BZP, MDPP and MeOPP) in the alternative in vivo model *C. elegans*. Besides, the effects of the derivatives in development, reproduction, behaviour, and modulation of neurotransmitters were analyzed.

2. MATERIALS AND METHODS

2.1. Chemicals

N-Benzylpiperazine (BZP, 99.3% purity) was purchased from Chemos GmbH (Regenstauf, Germany), 1-(3,4-methylenedioxybenzyl)piperazine (MDBP, 97% purity) from Aldrich Chemistry (Steinheim, Germany), and 1-(4-methoxyphenyl) piperazine (MeOPP, 96% purity) from Acros Organics (New Jersey, USA). 2′,7′-Dichlorofluorescein diacetate (DCF-DA) was provided by Sigma-Aldrich Co. (St. Louis, MO, USA). Bacto-agar anda bacto-peptone were obtained from Becton Dicknson BD (New Jersey, USA) and HiMedia Laboratories (Mumbai, India), respectively.

2.2 Strains and synchronization

N2 strains (wild-type) and transgenic nematodes with gene reporter BY200 [Pdat-1 :: GFP] were obtained through Caenorhabditis Genetics Center (CGC), (University of Minnesota, Twin Cities, MN, USA)

(http://www.cbs.umn.edu/CGC). The strains were maintained in Nematode Growth Medium (NGM) sown with *Escherichia coli* OP50 as feed supplementation and incubated at 20°C in incubator BOD.

The cultures of pregnant hermaphrodite *C. elegans* were synchronized through a lysis in alkaline solution (1% NaOCl, 0.25 M NaOH), followed by washing procedure with M9 buffer (0.02 M KH_2PO_4, 0.04 M Na_2HPO_4, 0.008 M NaCl, 0.001 M $MgSO_4$) and flotation of the eggs in a 30% sucrose (m/v) saturated solution to separate the maggot's eggs from the remainder bacteria, according to the procedures described by Brenner (Brenner, 1974). The eggs were incubated and left hatch to obtain worms at L1 larval stage in plaques with NGM without food from a day to other.

2.3 Exposure to designer drugs

Without using bacteria, the previously synchronized worms at L1 stage (2500 for LD_{50} determination, development of the worms and brood size, 1500 for measurement of reactive oxygen species (ROS) and fluorescence quantification) were exposed for 30 min (acute exposure) to 2.5 to 100 mM BZP, 1.0 to 25 mM MeOPP, or 0.25 to 5 mM MDBP, in liquid media containing 0.5% NaCl. After exposure, worms were washed three times with saline solution (0.5%) to remove the treatments. Stock solutions of BZP were made up in PBS. Stock solutions of MDBP and MeOPP were made in DMSO. In these cases, 5% DMSO in liquid medium was used as negative control. All stock solutions were stored at −20 °C and freshly diluted on the day of the experiment.

2.4 LD_{50} determination

For BZP, MeOPP and MDBP LD_{50} determination, the worms were exposed and washed according to the item 2.3. After, the worms were placed in NGM plates with OP50-seeded for food supplementation. The number of surviving worms on each plate was counted after 24 hours. The lethality was evaluated by normalizing the data as percentage of control. All drugs were tested in three independent experiments with each concentration tested in two replicates within each experiment.

2.5 Worm development test

For development evaluation, 48 hours after the acute exposure, 20 adult nematodes were photographed for measuring their body surface. For this, the NGM plates containing the treated worms were washed with distilled water, and the nematodes transferred to centrifuge tubes, being washed successively until the solution was free of bacteria. After, 15 µl of the solution containing the worms were deposited on a slide containing 2% agarose with 15 µl of 2.25% levamisole. Photos were taken, and the body area of the worms were measured manually using the software AxioVision LE (version 4.8.2.0 for windows). The results were expressed as percentage of body area in relation to the control group.

2.6 Measurement of reactive oxygen species (ROS)

After the exposure, worms were resuspended in 100 µL of 0.9% NaCl solution and transferred to 96-well plates. Additionally, 100 µl of 0.05 mM 2′,7′-dichlorofluorescein diacetate (DCF-DA) were added and the fluorescence levels were measured at a 90 minutes kinetic reading at a 10 minutes interval in a microplate reader (Spectramax Me2; Molecular Devices LLC, Sunnyvale, CA, USA) at 20ºC (excitation: 485 nm, emission: 535 nm). The values were expressed as percentage of fluorescence intensity relative to the control wells.

2.7 Drugs challenge for reproduction and behavioral tests

The experimental approach used was based on the individual dose-response curves of the drugs. For reproduction and behavioral tests, three concentrations were chosen: a non toxic concentration, a concentration near the LD_{50}, and a highly toxic concentration (above the LD_{50}). Based on this, it was selected 25 mM, 50 mM, and 65 mM concentrations for BZP, 2.5 mM, 5 mM and7.5 mM for MeOPP and the 0.5 mM, 1.0 mM and 2.0 mM for MDBP.

2.8 Brood Size

To evaluate the effects on reproduction, after 48 hours of acute exposure, four worms from each treatment of three selected concentrations were transferred separately to new OP50 NGM plates. The number of eggs laid were counted daily until the end of the reproductive period (4 days).

2.9 Behavioral test

2.9.1 Head thrash frequency

After 48h of exposure, individual worms were transferred to bacteria-free NGM plates. The movements of the head beat were counted for 1 min. A head thrash was defined as a change in the direction of flexion of the head relative to the body (Hu *et al.*, 2008). Four worms were analyzed from each treatment group, and the mean number of flexions was expressed. All drugs were tested in three independent experiments.

2.9.2 Nose touch

In addition to the head-trash frequency, the nose touch behavioral test was performed according to a previously described method (Murakami e Murakami, 2005). At intervals of 30s, a touch on the nose of each worm was performed and the response was analyzed according to three movements: forward sinusoidal movement (forwad), reversal movement (backward), and omega, which is when the head touches the tail resembling the Greek letter Omega. Four worms were analyzed from each treatment group and expressed on average by experiment. All drugs were tested in three independent experiments.

2.10 BY200 fluorescence microscopy

BY200 [Pdat-1 :: GFP] is a fluorescent strain that marks dopamine transporters (DAT), presented in neurons. After acute exposure, the worms

were transferred to 2% agarose pads with 2.25% levamisole and the fluorescence was observed in an epifluorescence microscope (Olympus, IX-71).

2.11 Statistical analysis

All statistical analyzes were generated with GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, USA). A sigmoidal dose-response model with 100% restriction was used to draw the curves and to determine the LD_{50} value. Normality of the data distribution was assessed by the Kolmogorov–Smirnov normality test. Statistical analysis of significance was performed by one-way ANOVA (for more than 2 groups) and two-way ANOVA was used for the ROS quantification followed by Bonferroni post-test. Significance was accepted at $p<0.05$.

3. RESULTS

3.1 Piperazine designer drugs present acute toxicity to *C. elegans*

Figure 2 shows the percentage of survival versus the tested concentrations of each piperazine. The concentration response curve analysis indicated that the LD_{50} for BZP, MeOPP and MDBP were respectively 52.21, 5.72, and 1.22 mM in N2 *C. elegans*. The LD_{50} obtained were very different among the three derivatives, indicating that the toxicity of the compounds might be related to the different substituents in piperazinic ring. All the tested doses were compared to the control group, which did not receive any treatment.

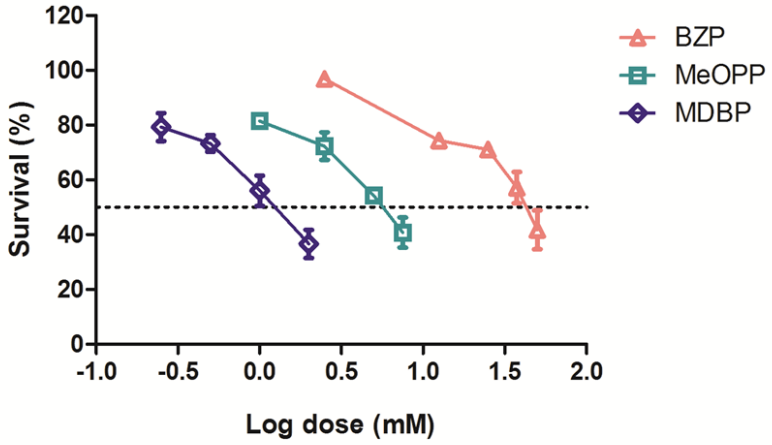


Figure 2. Log dose response curve for lethal dose 50% determination of piperazine designer drugs after acute treatment. BZP (N-benzylpiperazine); MeOPP (1-(4-methoxyphenyl)piperazine); MDBP (1-(3,4-methylenedioxybenzyl)piperazine). Values are expressed as means ± SEM from three independent experiments (n = 3).

3.2 Piperazine designer drugs affect the development of worms

The normal development of *C. elegans* was affected by exposure to the piperazine designer drugs. A significant reduction was observed in the body area of the worms when compared with control group in a concentration-dependent manner (p<0.001, Figure 3). For BZP (Figure 3A), a significant decrease in development was observed at 12.5 mM to 90 mM. At the highest concentration (90 mM), there was observed an approximately 50% reduction in the size of the worms. For MeOPP (Figure 3B), although this was not the most toxic compound, it was the derivative that most impacted the *C. elegans* development. The lowest concentration (1 mM) significantly reduced the worm size by 30% compared to the control, while a significant reduction of 82% was noted in the highest concentration tested. For MDBP (Figure 3C), a crescent reduction of size was observed. Starting with a 30% significant reduction in the lowest concentration (0.25 mM), and reducing up to 73% of the size of worms in the highest concentration (4 mM).

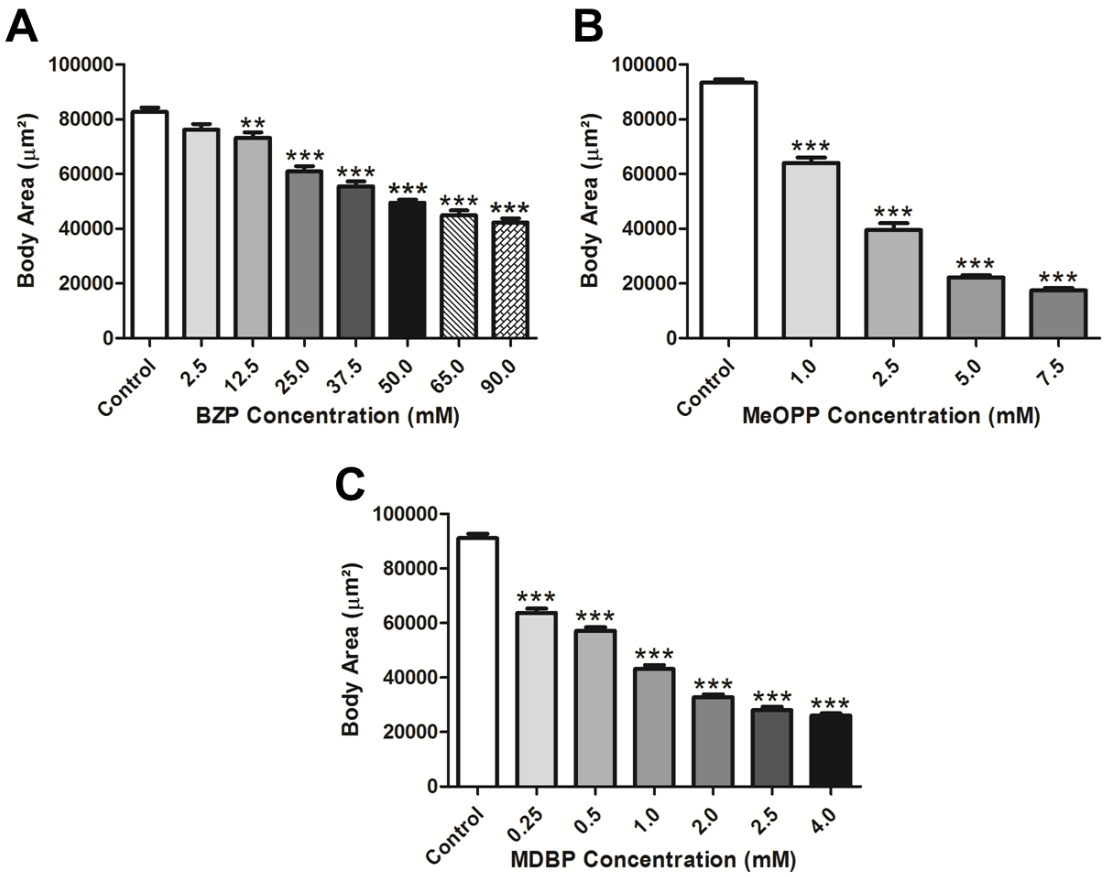


Figure 3. Body areas of *C. elegans* after acute treatment with piperazine designer drugs in different concentrations: (A) BZP, N-benzylpiperazine; (B) MeOPP, 1-(4-methoxyphenyl)piperazine; (C) MDBP, 1-(3,4-methylenedioxybenzyl)piperazine. Values are expressed as means ± SEM from three independent experiments (n=3). Statistical comparisons were made using one-way ANOVA/Bonferroni post-hoc test. (**p<0.01; ***p<0.001 vs control).

3.3 Piperazine designer drugs interfere in ROS production

ROS levels were determined with the dichlorofluorescein diacetate (DCF-DA), which is oxidized to the DCF fluorophore in the presence of free radicals. Surprisingly, there was a significant decrease (p<0.05, two-way ANOVA/Bonferroni) in ROS formation at all concentrations of BZP (Figure 4A). Differently, in MeOPP (Figure 4B) there was a significant increase (p<0.05, two-way ANOVA/Bonferroni) in ROS formation at 10 and 12.5 mM. In the MDBP

(Figure 4C), the increase of ROS was significant (p<0.01, two-way ANOVA/Bonferroni) only at the highest dose (4 mM).

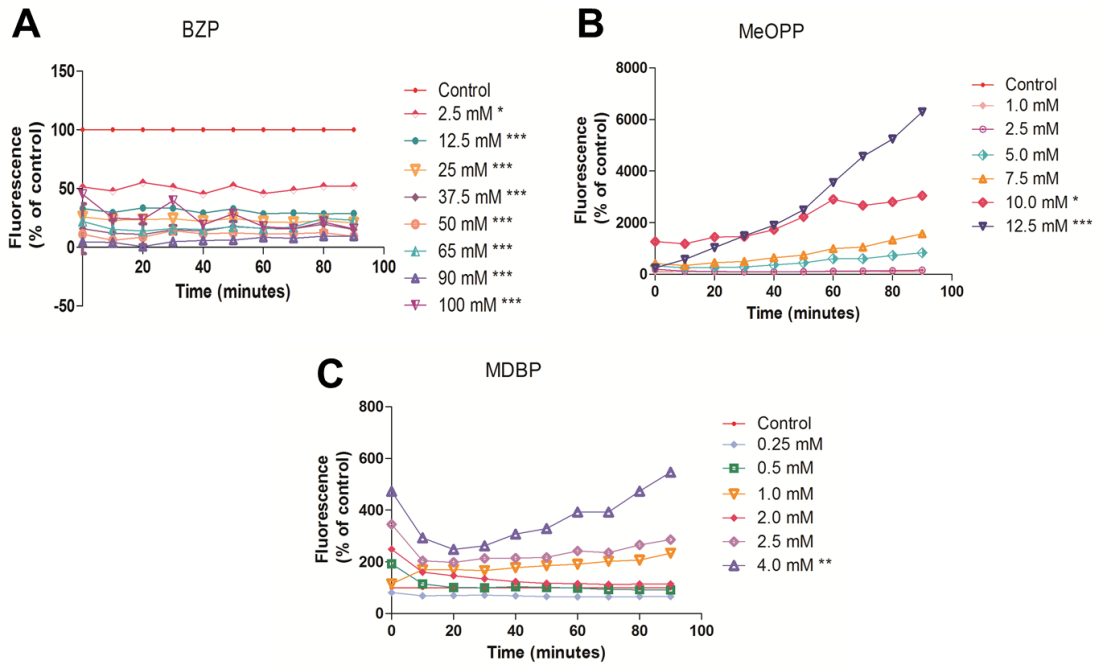


Figure 4. ROS levels measure by DCF-DA dye: (A) BZP, N-benzylpiperazine; (B) MeOPP, 1-(4-methoxyphenyl)piperazine; (C) MDBP, 1-(3,4-methylenedioxybenzyl)piperazine. Values are expressed as means ± SEM from three independent experiments (n=3). Statistical comparisons were made using two-way ANOVA/Bonferroni post-hoc test. (*p<0.05; **p<0.01; ***p<0.001 vs control).

3.4 Piperazine designer drugs decreased *C. elegans* reproduction

Figure 5 shows brood size of the nematodes after treatment with the piperazine designer drugs. For BZP (Figure 5A), there was a significant (p<0.01, ANOVA/Bonferroni) reduction in the number of eggs at 50 and 65 mM. Similarly, there was observed a significant reduction (p<0.05, ANOVA/Bonferroni) in brood size after treatment with 5.0 and 7.5 mM MeOPP (Figure 5B). For MDBP (Figure 5C), all tested concentration significantly reduced (p<0.001, ANOVA/Bonferroni) the brood size.

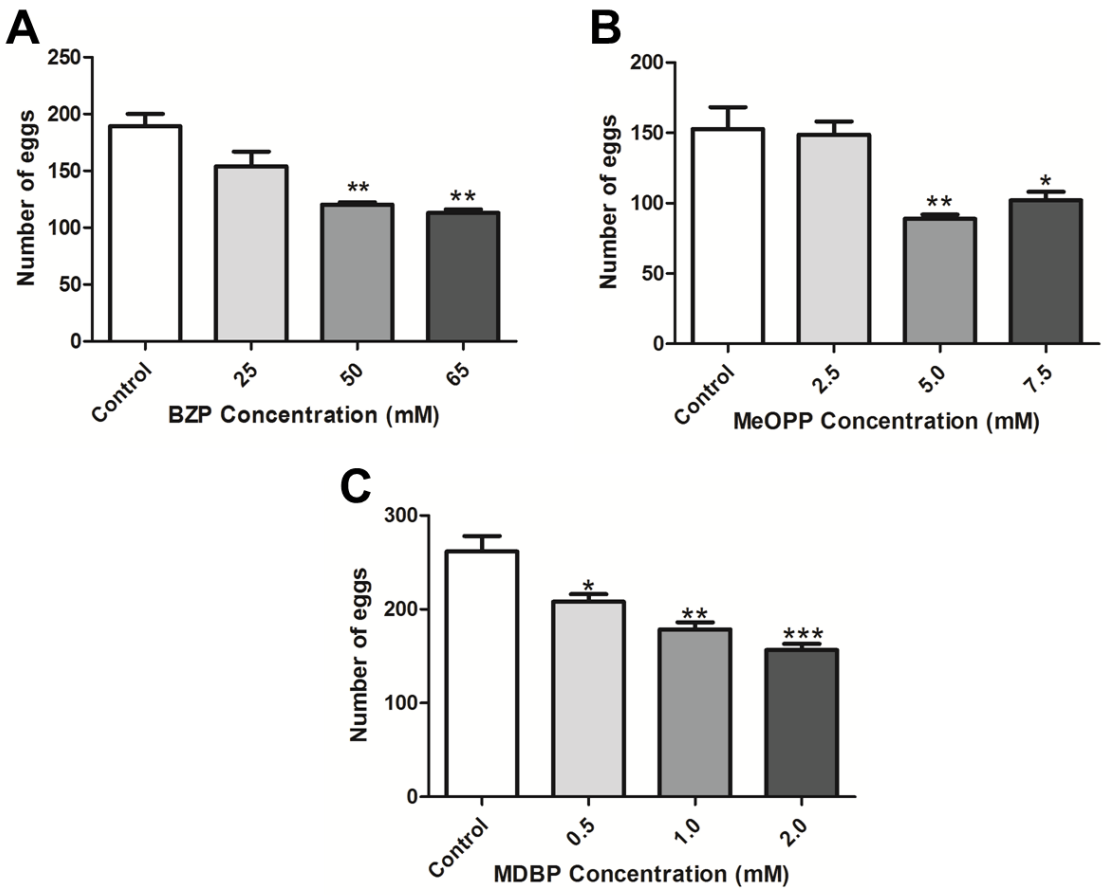
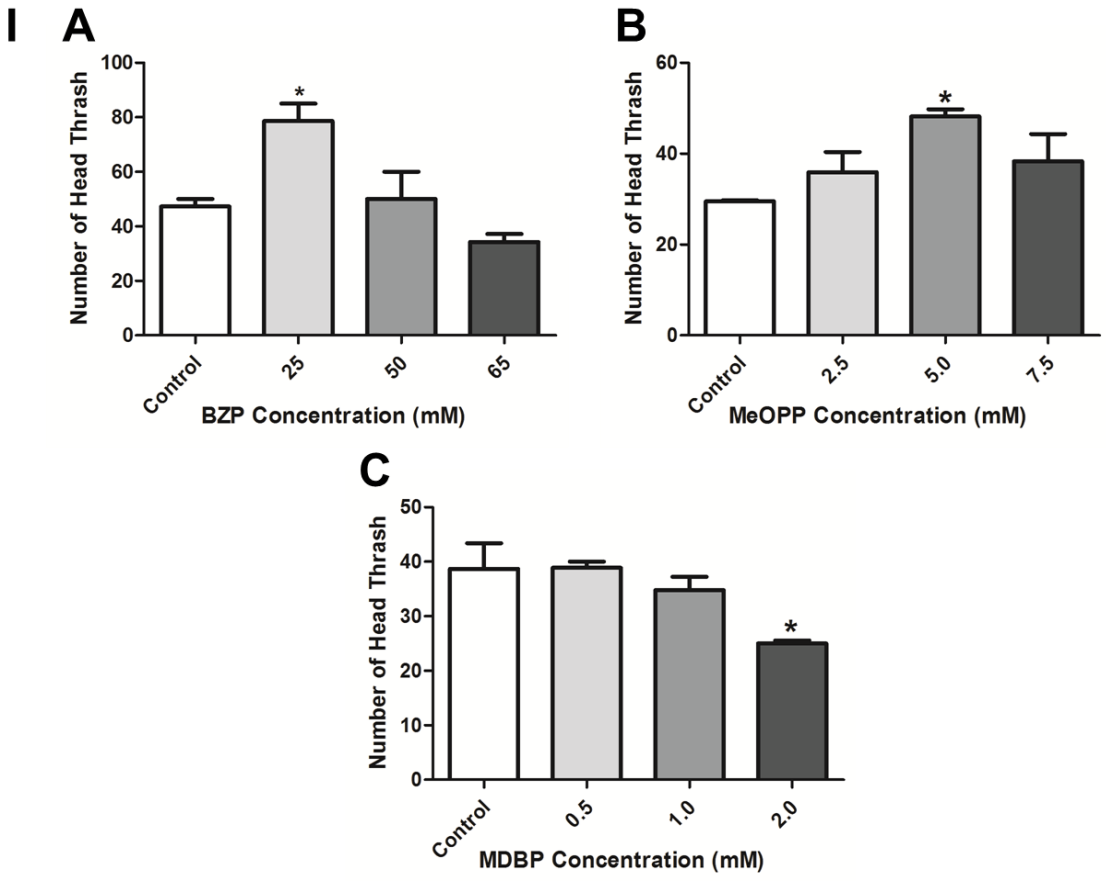


Figure 5. Effects of piperazine designer drugs on *C. elegans* brood size: (A) BZP, N-benzylpiperazine; (B) MeOPP, 1-(4-methoxyphenyl)piperazine; (C) MDBP, 1-(3,4-methylenedioxybenzyl)piperazine. Values are expressed as means ± SEM from three independent experiments (n=3). Statistical comparisons were made using one-way ANOVA/Bonferroni post-hoc test. (**p*<0.05; ***p*<0.01; ****p*<0.001 vs control).

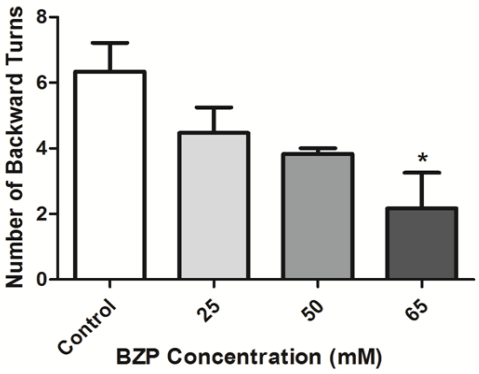
3.5 Piperazine designer drugs elicit changes in nematodes behavior

Figure 6 shows changes in nematode behavior after exposure. There was a significant (*p*<0.05, ANOVA/Bonferroni) increase in nematode locomotion, measured through the head thrashes, (Figure 6I) at 50 mM BZP (Figure 6-IA) and 5 mM for MeOPP (Figure 6-IB). Interestingly, for MDBP, there was a significant decrease (*p*<0.05, ANOVA/Bonferroni) in locomotion at 2 mM (Figure 6-IC).

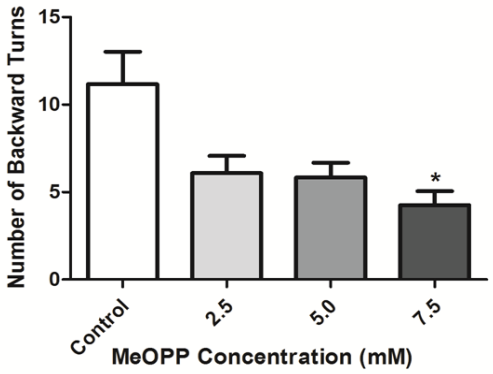
In the nasal touches, required for neuronal desensitization, it was possible to observe a significant decrease of movements (p<0.05 and p<0.001) in the last concentrations of all molecules tested (Figure 6II and 6III), and increase in omega movement (Figure 6IV) (Figure 6IVC, p<0.001) at 1 and 2 mM for MDBP (Figure 6IVC, p<0.001).



II A



B



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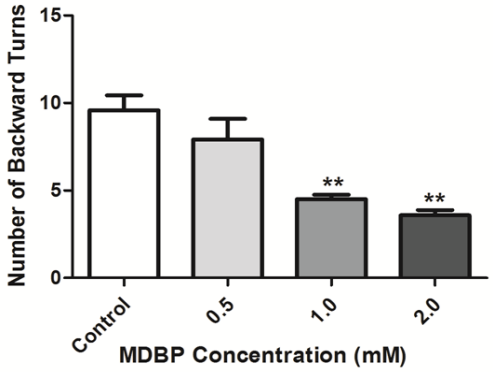
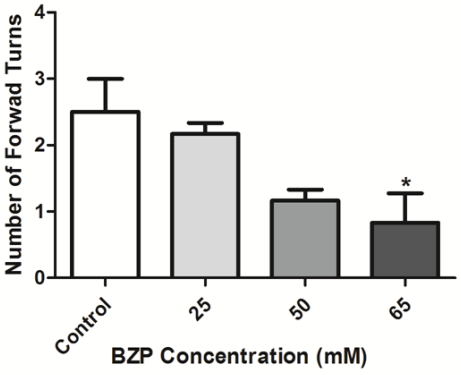


III A



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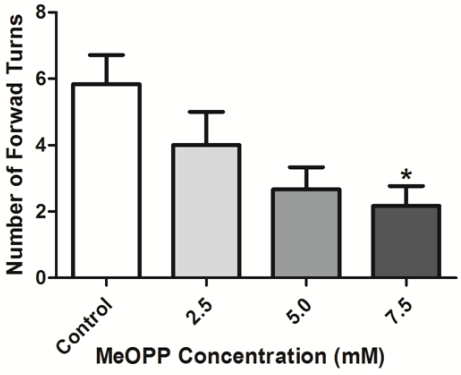

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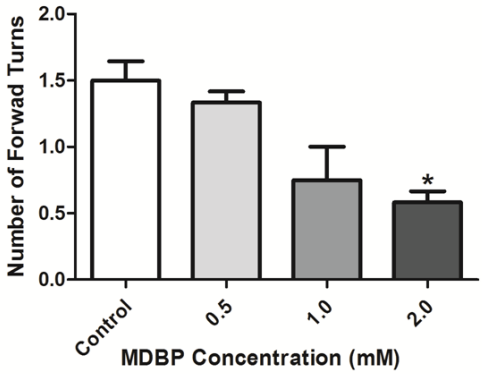


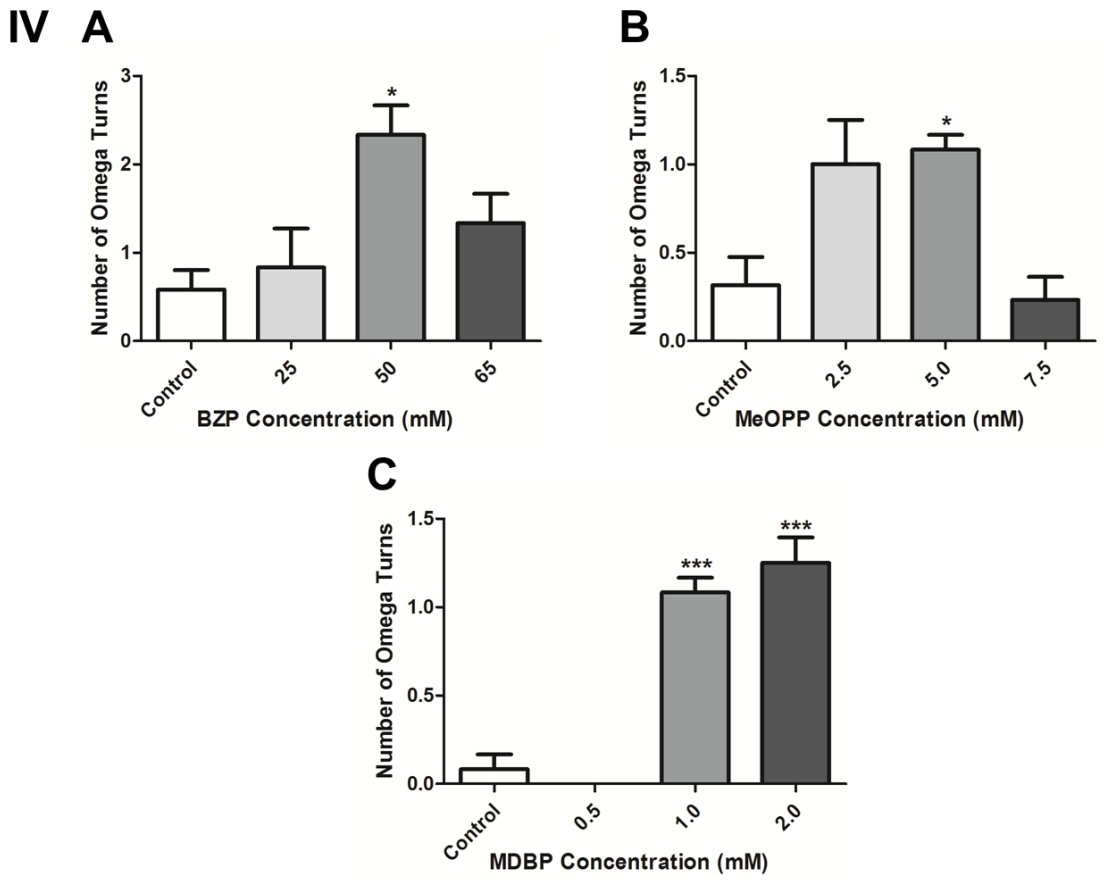
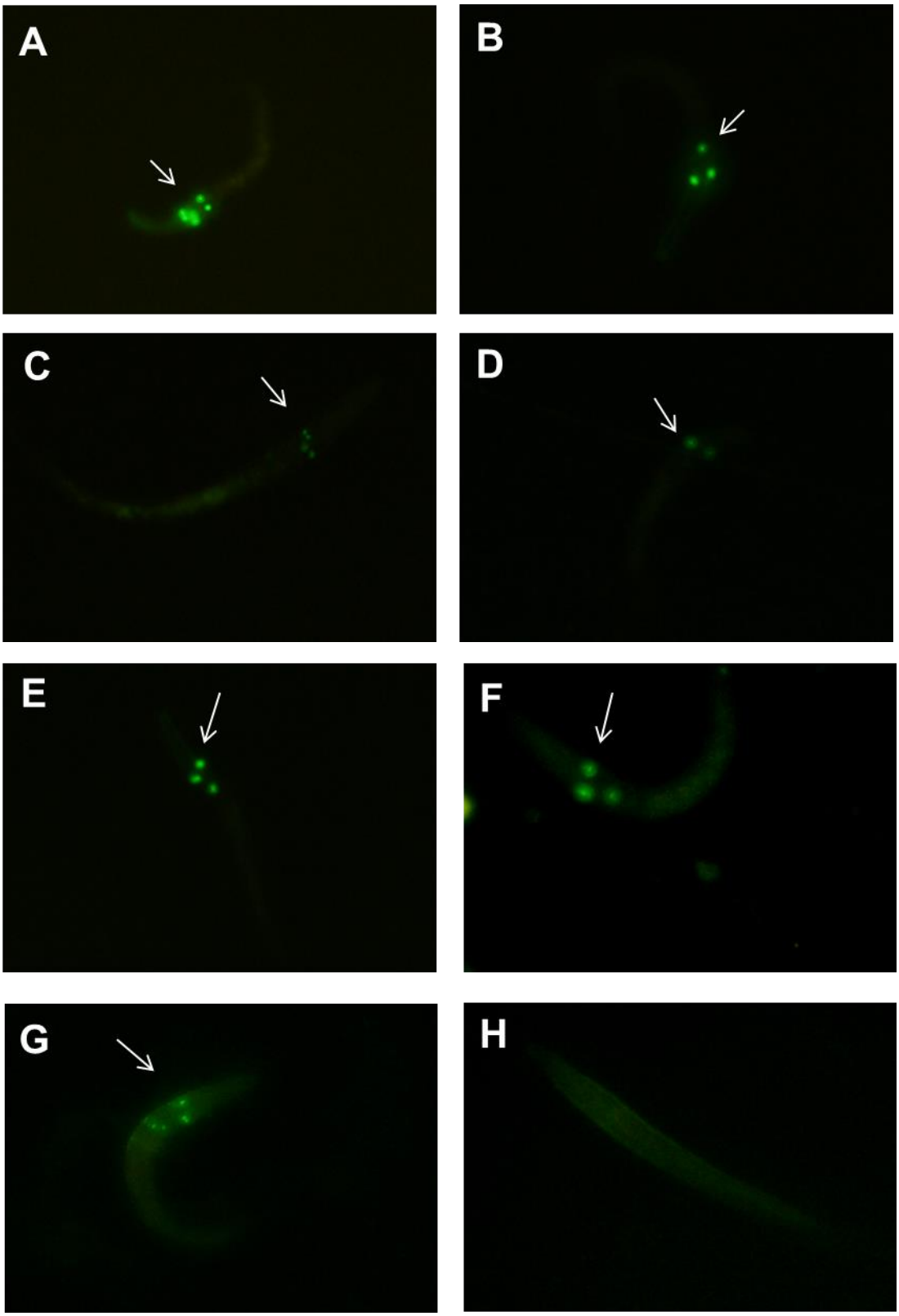


Figure 6. Acute exposure to piperazine designer drugs promotes a decrease in *C. elegans* basic movements. (I) Head thrashes frequency. (II) Backword turns. (III) Forwad turns. (IV) Omega turns. (A) BZP, N-benzylpiperazine; (B) MeOPP, 1-(4-methoxyphenyl)piperazine; (C) MDBP, 1-(3,4-methylenedioxybenzyl)piperazine. Values are expressed as means ± SEM from three independent experiments (n=3). Statistical comparisons were made using one-way ANOVA/Bonferroni post-hoc test. (**p*<0.05; ***p*<0.01; ****p*<0.001 vs control).

3.6 Dopaminergic neurons are desensitized by piperazine designer drugs

The damage to dopaminergic neurons was investigated using the transgenic strain BY200 (dat-1p::GFP; rol-6), which expresses the green fluorescent protein at DATs. Piperazine designer drugs caused a dose-dependent neuronal damage, reflected by discontinued and punctuated GFP fluorescence as it can be visualized in figure 7.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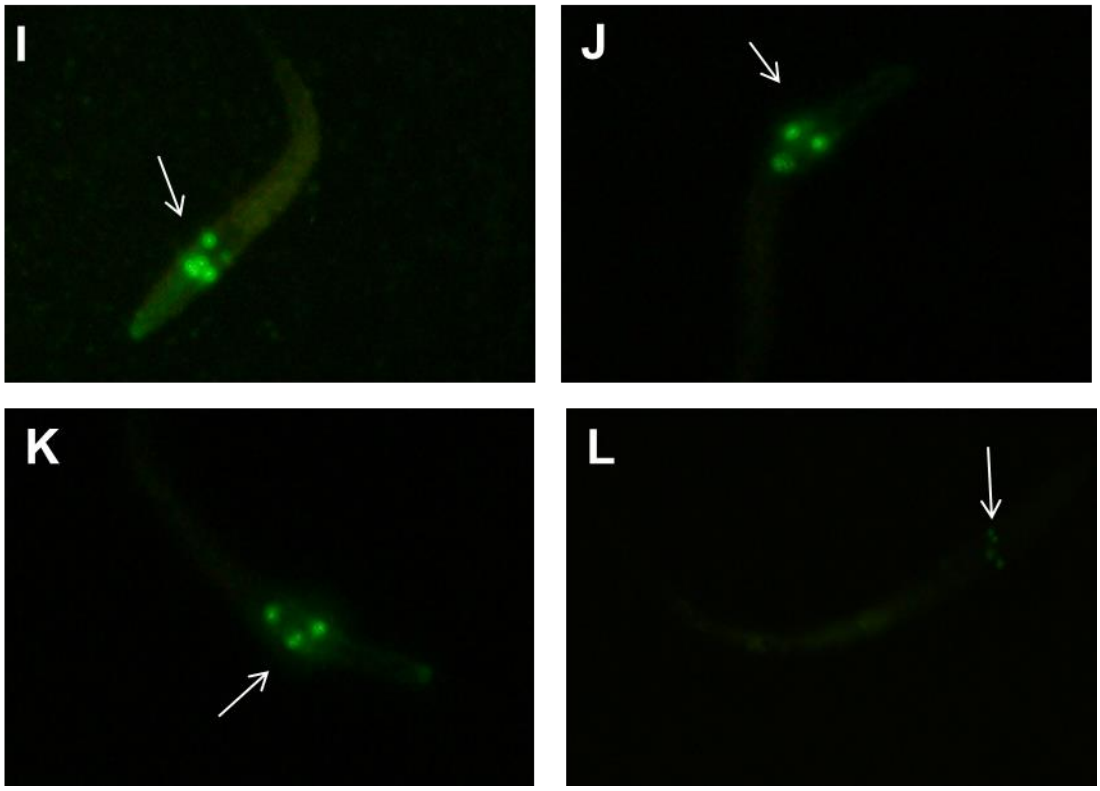

Figure 7. Dopaminergic neurons in alive L4 *C. elegans* (Pdat-1::GFP) following piperazine designer drugs exposure. Morphological changes were evident with all the compounds concentrations, and arrows indicate shrunken soma and puncta in the dendrites. (A)(E)(I) Control group; (B) BZP, N-benzylpiperazine (25mM); (C) BZP, N-benzylpiperazine (50 mM); (D) 65 mM. (F) MeOPP, 1-(4-methoxyphenyl)piperazine (2.5 mM); (G) BZP, N-benzylpiperazine (5.0 mM); (H) BZP, N-benzylpiperazine (7.5 mM); (J) MDBP, 1-(3,4-methylenedioxybenzyl)piperazine (0.5 mM); (K) MDBP, 1-(3,4-methylenedioxybenzyl)piperazine (1.0 mM), and (L) MDBP, 1-(3,4-methylenedioxybenzyl)piperazine (2.0 mM);

4. DISCUSSION

The present study focused on three piperazine derivatives consumed for recreational purposes. In order to exert their pharmacological action in the central nervous system, they must cross the blood-brain barrier. Therefore, their central activity is determined by the speed and extent to which they can

penetrate nerve tissue, being related to the lipophilic characteristics of the compounds, their binding to plasma proteins and their ionization constant (Escada, 2007).

The absorption of substances is an essential prerequisite for their systemic effects. The nematode cuticle is a barrier to absorption, protecting them from exposure to toxic substances. Therefore, the oral exposure is the preferred route for absorption of xenobiotics in nematodes. Charao *et al(2015)* demonstrated for the first time the internalization and distribution of drug-carrying nanoparticles in *C. elegans*, using as strategy the labeling of these particles with rhodamine B conjugate. In liquid exposures, nematodes are forced to swim and orally absorb the dissolved compounds.

Among the three piperazine designer drugs studied, BZP showed the lowest toxicity (52.21 mM). In humans the BZP is not extensively metabolized (Escada, 2007). It has been described that 5 to 30% of the drug is excreted in its non metabolized form (Parkin *et al.*, 2004). In contrast, MeOPP and MDBP were the piperazines which showed the highest toxicity (5.72 mM and 1.22 mM, respectively). In humans these two derivatives are extensively metabolized (Escada, 2007). Using Wistar rats as a model, the most important metabolic process for MeOPP is O-demethylation of its methoxyl group. The formed product, 1-(4-hydroxyphenyl)piperazine, is subsequently conjugated with glucuronic acid and sulfates (Staack e Maurer, 2005). In MDBP the metabolic process was studied qualitatively in male Wistar rats (Maurer *et al.*, 2004). The main route is the demethylation of the 3,4-methylenedioxy portion for catechol and additional for N-(4-hydroxy-3-methoxybenzyl)piperazine followed by partial glucuronidation or sulfation followed by N-alkylation.

For MeOPP and MDBP, the main metabolic reactions are the demethylenation and the demethylation, respectively, which are catalyzed by cytochrome P450 enzymes (CYPs). Among the several isoenzymes, CYP2D6 was considered the main responsible for these reactions (Staack *et al.*, 2004) Other isoenzymes also involved with piperazine designer drugs metabolism are CYP3A4, CYP1A2 and CYP2C19 (Antia *et al.*, 2009). Using primary rat hepatocytes as in vitro model, the piperazine designer drugs were co-incubated with metyrapone (general CYP450 inhibitor) and quinidine (specific CYP2D6 inhibitor), showing a deviation to the left in relation to the curves obtained with

the drugs with no inhibitors. This indicated that the CYP450-mediated metabolism has a detoxifying effect, and it is very likely that the parent substances are more toxic than their metabolites (Dias-Da-Silva *et al.*, 2015).

According to the WormBook, *C. elegans* presents in its metabolic process the CYP450 enzymes, and among them the CYP2D6 isoenzyme. In addition, the nematodes also show homology with the COMT gene that metabolizes catecholamines. This suggests that the worm presents a metabolic pathway very similar to that presented by the primary rat hepatocytes and, possibly, the humans.

The neurotoxicity of piperazine designer drugs was evaluated in human neuroblastoma SH-SY5Y cells differentiated cells for a dopaminergic phenotype. In this study, MDBP showed higher toxicity (EC50 = 144 µm), followed by MeOPP (EC50 = 274 µm) and BZP (EC50 = 721 µm) (Arbo *et al.*, 2016). Interestingly, in the present *in vivo* study using *C. elegans* as in vivo model, the same toxicological profile was observed, corroborating this results and showing a potential application of *C. elegans* in translational toxicological studies.

The development of the worm was monitored by the measurement of body area. The growth of *C. elegans* is determined by a conservative pathway of genetic regulation and it is a good parameter to evaluate toxic effects (Wu *et al.*, 2013). In the present study, the reduction in the body area of the worms after exposure to piperazine designer drugs is an indicative of neurotoxicity. The C. elegans body size is is influenced by environmental conditions, and the nervous system plays a crucial role in this process. Besides, neurotransmitters, such as dopamine, regulate body size affecting muscle function as they affect the activity of motor neurons (Nagashima *et al.*, 2016). Considering that piperazine designer drugs could act at noradrenergic, dopaminergic and serotonergic synapses, it is very possible that the reduction of body area is connected to dopaminergic effects. Indeed, nematodes exposed to piperazine and piperazine analogs showed a slight delay in development, compared to untreated worms (Racz *et al.*, 2017).

Oxidative stress, which occurs when ROS generation leads to an imbalance between ROS and antioxidants, is involved in the mechanism of toxicity of a large number of compounds (Sies, 1997). It is a well described

mechanism that plays an essential role in the toxic effects of various amphetamine derivatives that induce the formation of highly reactive species (Barbosa *et al.*, 2014; Silva *et al.*, 2014). Interestingly, piperazine designer drugs did not induce ROS formation in the *in vitro* models using rat cardiomyoblasts H9c2 (Arbo *et al.*, 2014) and human neuroblastoma SH-SY5Y cells (Arbo *et al.*, 2016), but increased oxidative stress in primary rat hepatocytes (Dias-Da-Silva *et al.*, 2015). In the present study, the highest MeOPP and MDBP concentrations increased ROS production, no alterations were seen for BZP. Life span is regulated by prooxidant/antioxidant homeostasis in worms (Halliwell e Gutteridge, 1984). The results suggest that oxidative stress probably is not the main mechanism of toxicity in piperazine designer drugs, but it is related, at least in part, with the effects of these drugs.

Piperazine designer drugs also altered the *C. elegans* reproductive process. Worms showed decreased egg laying after exposure to BZP, MeOPP and MDBP, which might indicate the late development of animal gonads, as demonstrated in other studies with other metal compounds (Hu *et al.*, 2008) The piperazine designer drugs effects are related to the amphetamine drugs, which are mediated by dopamine in *C. elegans* (Carvelli *et al.*, 2010). Interestingly, both MDMA and methamphetamine, other group of designer drugs of abuse, also had an effect on egg laying, decreasing litter size and egg numbers when worms were exposed to these recreational drugs (Schreiber e Mcintire, 2011). Furthermore, piperazine and piperazine analogs showed the highest sensitivity in affecting brood size in nematodes (Racz *et al.*, 2017).

The in vivo model *C. elegans* presents advantages related to the neurotoxicological evaluation due to the particularly well characterized nervous system (Leung *et al.*, 2008). With 302 neurons representing 118 neuronal subtypes characterized (Hobert, 2005), *C. elegans* provides a good experimental model for studying neuronal lesion mechanisms with resolution of individual neurons. This relatively simple nervous system consists of 6393 chemical synapses, 890 electrical and 1410 neuromuscular junctions (Chen *et al.*, 2006). In addition, the main neurotransmitter systems and their genetic networks are conserved phylogenetically from nematodes to vertebrates, which allows the findings in *C. elegans* to be extrapolated and still confirmed in vertebrate systems.

Our data showed locomotion defects and mechanical sensitization after 48 h of exposure to piperazines designer drugs. In invertebrates, changes in movement are the most common points for determining behavioral effects after toxic exposures (Anderson *et al.*, 2004). In *C. elegans*, when neuronal functions are impaired, changes in behavior may appear (De Almeidaáfagundez *et al.*, 2015). The treated worms showed decreased motor function and reduced mechanical sensitivity, which strongly suggests that these drugs might induce neuronal damage. The mechanosensory functions of the dopaminergic and serotonergic neurons are evaluated by observing the ability of worms to decrease the speed of locomotion in an environment without food (Leung *et al.*, 2008). The head thrash movement shows the locomotive capacity of the nematode when it is placed in an environment where it needs to go in search of food. Our results show that the lower dose of BZP and intermediary dose of MeOPP designer drugs stimulated the worm, increasing its locomotion rate, whereas at the highest concentration, MDBP demonstrated neurotoxicity, reducing the locomotor capacity of the worm in a new environment. *C. elegans* responds initially to an unlocated mechanical stimulus or a gentle touch on its anterior body (touch), by rapidly reversing the direction of movement (Rankin *et al.*, 1990). The worms treated with piperazine designer drugs lost their dose-response style stimulus as the concentration increases, also as a sugn of neurotoxicity. We can see that when the worms are treated with the piperazines, they lose the stimulus as the concentration increases, again indicating neurotoxic parameters.

The dopaminergic system in *C. elegans* is particularly simple to visualize using the DAT-1 :: GFP strain. Containing only eight dopaminergic neurons in the hermaphrodite, these neurons can be observed after exposure to toxic substances (Sulston *et al.*, 1975). Accordingly, we have found that GFP-tagged DAT showed lower fluorescence after exposure to piperazine designer drugs. The lost of fluorescence strongly indicates neurodegenerative changes and neuronal death, showing the neurotoxic potential of these drugs.

In combination, our results show that toxicity to piperazine designer drugs can cause serious damage by altering neuronal function, characterized by behavioral and morphological changes in *C. elegans.*

5. CONCLUSIONS

In conclusion, we first describe the toxicity and neurotoxic potential of piperazine designer drugs in the alternative model in vivo *C. elegans*. Among the derivatives tested, MDBP was the most potent in inducing toxicity. Worms acute exposure to piperazine designer drugs resulted in multiple biological defects, including changes in survival, development, reproductive and locomotor impairment, which might be related to dopaminergic neuronal damage, as well as motor function and reduced mechanical sensitivity. Therefore, additional studies should focus on transgenic knockout strains to compare the dopaminergic effects involved in the toxicity mechanism of piperazine designer drugs.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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