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Behavioral and dopaminergic damage induced by acute iron toxicity in *Caenorhabditis elegans*

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Abstract

Iron (Fe) is an important metal to the organism homeostasis and exists abundantly in the environment. Moderate levels of Fe obtained from food are necessary for normal cell physiology; however, abnormally high levels of Fe may have toxic effects by reducing H_2O_2 to the highly cytotoxic hydroxyl radical (OH•) (Fenton catalysis). Fe is ubiquitous toxicant to the environment and also widely used in food products, however its effects to the nervous system are not well understood. Herein, we evaluated the toxic effects of Fe using *C. elegans* and investigated various parameters in order to contribute to the understanding of Fe-induced toxicity and to validate this model. Our goal was to search for therapeutic targets that are more effective than those currently used. The Fe LD_{50} of acute exposure (30 min) was 1.2 mM, and we verified that worms readily take up this metal. Furthermore, sublethal Fe concentrations significantly decreased the worms' lifespan and brood size compared to non-exposed worms. We also observed that animals exposed to Fe had decreased locomotor activity and decreased mechanic sensitivity, suggesting possible dysfunction of the nervous system. In agreement, we found cholinergic and dopaminergic alterations in the worms. In summary, we suggest that Fe leads to selective neuronal damage, which might be the underlying cause of altered behavior and reproductive defects.

Keywords: iron toxicity, oxidative stress, locomotion behavior, acute exposure, Caenorhabditis elegans

1. INTRODUCTION

Iron (Fe) is an important metal for aerobic cell physiology and exists abundantly in the environment. As a micronutrient, iron plays a fundamental role as a component of mitochondrial respiratory chain complexes [1]. Consequently, Fe deficiency can be associated with abnormal neuroembryogenesis, myelination and metabolism of biogenic amines. On the other hand, abnormally high levels of Fe can cause toxic effects in many organisms[2] through acute intoxication in children or chronic exposure, especially caused by environmental contamination. Fe is a structural component of catalase and oxygen storing or transporting proteins (myoglobin and hemoglobin). In general, most of the absorbed Fe is bound to storage or transporting proteins and the levels of intra and extracellular free Fe are very low. Indeed, even low levels of free Fe can cause toxic effects in different types of cells [2]. In agreement, a vast amount of data indicates that Fe is an important etiologic factor associated to oxidative stress induction and cell demise in pathological situations [3-6]. It is known that Fe accumulation causes free radical damage through the Fenton reaction [7, 8], as Fe^{2+} reduces hydrogen peroxide to the highly-cytotoxic hydroxyl radical (OH•) (Fenton catalysis). Fe-induced oxidative stress is thought to be involved in the pathogenesis of some neurodegenerative diseases, such as Parkinson´s disease [1, 9] as Fe deposition is increased in brain regions of subjects affected by these chronic degenerative diseases [10-12].

Accordingly, it is imparative to evaluate the toxicity of Fe to the nervous system. I*n vitro* studies are quite limited in scope, as they fail to reflect absorption and distribution of metals *in situ*. *Caenorhabditis elegans*, a free-living soil nematode, is an optimal model for toxicity testing and an alternative method to replace mammalian models [13,14, 15]. *Caenorhabditis elegans* is well-characterized at the genetic, physiological, molecular and developmental levels [16]. It was the first multicellular organism to have its genome completely sequenced, which has been found to have a high level of conservation with the vertebrate genome [17-19].

Advantages of the worm vs vertebrate models include short life cycle, small size, easy cultivation and behavioral assessment. The nervous system of the *C. elegans* is composed of 302 neurons [20], which can be specifically marked with green fluorescent protein (GFP) and observed under fluorescence microscopy. In addition, behavioral evaluation can be assessed, aiming to analyze neuronal functioning. C. elegans has 8

dopaminergic neurons that control several functions such as movement, feeding and defecation. Hence, these parameters can be used for behavioral evaluation.

In this study, we evaluated the toxic effects of Fe using *C. elegans* by assessing different endpoints of toxicity. Our goal was to contribute to better understanding on Fe-induced neurotoxicity. We hypothesized that Fe causes changes in egg-laying, longevity and locomotion, leading to neuronal neurodegeneration even upon a short exposure time to Fe.

2. MATERIALS AND METHODS

Chemicals and Strains

All *C. elegans* used were originally obtained from the Caenorhabditis Genetics Center (CGC). The *C. elegans* strains Bristol N2 (wild-type), CF1553 (muls84), GA800 (wuls151) and BY200 (*dat-1*::GFP) were maintained on nematode growth medium (NGM) plates seeded with Escherichia coli OP50 at 20ºC [19]. Gravid *C. elegans* were washed off the plates into centrifuge tubes and were lysed with a bleaching mixture (0.45N NaOH, 2% HOCl) to obtain a synchronous populations of L1 (first larval stage) after ±14 hours, when eggs hatched [21]. Fe sulfate ($FeSO_4$), bacto-agar, bacto-peptona and other reagents were obtained from Sigma Aldrich.

Fe exposure

In the absence of bacteria, 1,500 L1 worms previously synchronized were exposed for 30 min to Fe concentrations of 0.05; 0.1; 0.5; 1.0; 1.5 and 2.0 mM in liquid media containing 0.5% NaCl. After exposure, worms were washed 3 times with saline solution (0.5%) and then transferred to NGM recovery plates inoculated with Escherichia coli/OP50 to posterior assays.

LD50 determination

To determine the lethal $FeSO_4$, worms were exposed as described above and the alive worms were counted 24 hours after exposure. The lethality was evaluated by normalizing the data as percentage of control. Three replicates were performed.

Fe levels measurement by GFAAS

After Fe exposure (triplicates), samples containing 10,000 worms were treated as described above. After six washes with saline solution (0.5%) worms were frozen and 24hs later they were dried at 80ºC for 4h. Then it was added 250 µL of nitric acid (P.A) and samples were left in water bath at 70º C for 1h. The Fe levels were quantified by Graphite Furnace Atomic Absorption Spectrometry (GFAAS).

Reproduction (egg laying)

To evaluate the Fe effects on reproduction, three worms from each Fe treatment were transferred separately to new NGM/OP50 plates every day. The eggs were counted daily until the end of the reproductive period.

Lifespan

Twenty L4 worms from each group were transferred to new NGM/OP50 plates every day and scored until all animals were dead. The nematodes were considered dead if they did not respond to a mechanical stimulus using a small wire [22]. All the experiments were made in duplicates and repeated at least three times.

Head thrash frequency

The assays were performed 48 hours after Fe exposure. Individual worms were transferred to a NGM/*E. coli* free plate. After 1 min recovery period, the head thrashes were counted for 1 min. A thrash was defined as a change in the direction of bending at the mid body [2]. We analyzed five worms from each group considered the mean number of head trashes per experiment. Each experiment was repeated 3 times.

Nose touch

The analysis was performed as previously described [23] and worms were observed 48hs after Fe exposure. Three basic movements were measured in a 30-s interval: forward sinusoidal movement (forward turns), reversal movement (backward turns), and Omega/U turns. In Omega turns, the nematode's head touches the tail and its shape looks similar to the shape of Greek letter Omega, whereas the angle of the

152 body bend is typically > 90◦ in U turns [24]. We analyzed 5 worms of each group at every
153 experiment and calculated the average per experiment. Each experiment was repeated 3
154 times.

155 **Egg-laying induced by Levamisole**

156 After Fe exposure, worms were transferred to plates with levamisole (1mM), an
157 anthelmintic which functions as a cholinergic agonist, for 30 minutes. The occurrence of
158 whole-worm hypercontraction and increased laid eggs were counted [25]. Worms with
159 functional neurotransmitter release should be sensitive to the substance lay more eggs
160 due to the hypercontraction [25]. Resistance to levamisole can typically indicate a post-
161 synaptic defect.

162 **Dopaminergic neurons images**

163

164 BY200 (Pdat-1::GFP) L4 later exposed animal were transferred to agarose (2%)
165 pads in M9 with 22.5 µM of levamisole. Image acquisitions were carried out with an
166 epifluorescence microscope in an appropriated room (20-22°C).

167

168 **ROS levels**

169 After iron exposure, L1 worms or L4 were transferred to a 96-well plates and
170 50uM of 2′7′ dichlorodihydrofluorescein diacetate (H_2DCF-DA) was added and their
171 fluorescence levels (excitation: 485 nm; emission: 535 nm) were detected using a
172 Multidetection microplate reader Chameleon (HIDEX) heated at 20°C. The
173 fluorescence from each well was measured every 10 min for up to 1.5 h. Here, we report
174 values obtained at 1 h. Fluorescence measurements were expressed as percent control.
175 Measurements were repeated 3 times, each condition was performed in duplicate.

176

177 **Statistical analysis**

178 All statistical analysis and figures were generated with GraphPad Prism
179 (GraphPad Software Inc.). We used a sigmoidal dose-response model with a top
180 constraint at 100% to draw the curves and determine the LD50 value reported in the
181 graph. Statistical analysis of significance was carried out by one-way ANOVA (for
182 more than 2 groups) followed by Tukey post-hoc test when appropriated. For longevity,

a repeated measure ANOVA was used in order to analyze the whole curve. It was considered significant when $p \leq 0.05$. Error bars represent standard error of mean (SEM). For the quantification of neurons fluorescence we used the software ImageJ and then transferred the data to GraphPad.

RESULTS

Fe acute exposure in *C. elegans* decreases worms survival and reproduction

The dose-response curve analysis indicated that the LC_{50} for $FeSO_4$ was 1.2 mM in wild-type worms (Figure 1). Following this assay, we selected the $FeSO_4$ concentrations of 0.05mM, 0.1mM and 0.5mM to study Fe toxicity. All the tested Fe concentrations significantly reduced worms lifespan compared to control (Figure 2, $p<0.05$). In addition, we observed that animals acutely exposed to Fe showed decreased total brood size (Figure 3, $p<0.05$). In adition, immediately after Fe exposure, worms showed increased ROS levels (Figure 8A, $p<0.05$), which were still elevated in L4 worms (Figure 8B, $p<0.05$).

Fe levels increased in a concentration-dependent manner

The Fe content was measured to verify whether worms were absorbing the metal in a dose-dependent manner. Figure 4 shows that Fe deposition in *C. elegans* increased as a function of the Fe dose, with a plateau reached at about 1mM.

Changes in nematodes behavior may indicate neuronal damage

There was a significant decrease in locomotion (Figure 5A, $p<0.05$) and in the nose touches necessary to neurons habituation (Figures 5B and C, $p<0.05$) 48hs after acute Fe exposure. In the levamisole paralysis assay Fe also led to decreased muscular contraction as seen by the egg laying (Figure 6).

Dopaminergic neurons are sensitive to Fe acute exposure

We investigated possible dopaminergic neurons damage using a transgenic strain BY200 (*dat-1*p::GFP; rol-6) which expresses the green fluorescent protein in these especific neurons. Fe caused a dose-dependent neuronal damage, reflected by discontinued and punctuated GFP fluorescence, as well as shrunken soma and reduced soma fluorescence (Figure 7).

DISCUSSION

Fe is an essential micronutrient for different types of living cells, especially for proteins that depend on this metal to be active. However, even small concentrations of free Fe can cause cytotoxic effects. Fe is essential for mammalian homeostasis (recommended daily allowance for humans is 10-15mg Fe) and at higher levels (20-30 mg Fe) it may become toxic [26, 27]. Several experimental studies on Fe toxicity have been carried out in mice, rats and humans [28, 29], however these models are complex, have long life span and the observation of neuronal viability *in vivo* is difficult to characterize. Herein, we found that acute Fe exposure in worms caused alterations in survival, life span and reproduction, and increased ROS levels; furthermore, Fe-treated animals showed decreased motor function and reduced mechanic sensitivity, which strongly suggest that Fe caused neuronal damage. Notably, we observed that both the dopaminergic and cholinergic systems were affected.

We used acute oral exposure to Fe in first larval staged worms. It has been shown that L1 worms are more sensitive to the reproductive toxicity than young adult nematodes, and may be more sensitive to reproductive and other endpoints of toxicity[30]. The nematodes' cuticle is a barrier for the absorption, protecting them from exposure to toxicants. Accordingly, oral exposure was deemed the preferred route for Fe absorption. In liquid exposures the nematodes are forced to swim and continuously absorb orally dissolved Fe^2. Living organisms have developed specialized mechanisms to tightly regulate iron uptake, storage and efflux, as well as *C. elegans*. The nematode expresses orthologs of key human \ genes and pathways that regulate iron metabolism[31]. Worms can regulate the intake of Fe through feeding or by altering the expression of homologous ferritin (ftn-1 and ftn-2), DMT1 (smf-1, smf-2 and smf-3) and/or ferroportin (fpn-1.1, fpn-1.2 and fpn-1.3) genes [32]. Remarkably, we confirmed Fe absorption in the treated worms by GFAAS quantification, and the total content of Fe in the worms increased to a maximal value in the 1mM group.

The worms showed decreased egg-laying following Fe exposure, which may indicate delayed development of animal gonads, as demonstrated in other studies with other metals/compounds [2, 33]. Taking into account that the reproductive system is closely linked to longevity in *C. elegans* [24], we verified that lifespan was also reduced in Fe-

exposed worms when compared to controls. Lifespan is regulated by the prooxidant/antioxidant homeostasis in worms. Fe is a classic inducer of reactive oxygen species (ROS) *in vitro* and *in vivo*, via the Fenton reaction. Fe (II) is oxidized by H_2O_2 to Fe (III), generating the highly reactive hydroxyl radical[26, 34]. The Fe-catalyzed Fenton reaction is a major source of hydroxyl in biological systems[35, 36] though other transition metals (e.g. copper) can also catalyze this reaction. In agreement, we corroborated that Fe increased ROS formation in worms and also reduced the lifespan concomitant with impaired reproduction.

Our data showed defects in locomotion and mechanic sensation after 48h of Fe exposure[2, 22, 24, 37]. Previous data suggest that ROS generated by Fe toxicity might be involved in neuron damage [38]. In invertebrates, changes in movement are the most common endpoints for determining behavioral effects after toxic exposures [37]. In *C. elegans,* when neuronal functions are impaired changes in behavior may appear. In agreement, we have found that GFP-labeled dopaminergic neurons showed decreased fluorescence after Fe exposure, indicating neurodegenerative changes. Furthermore, we observed aberrant functioning of cholinergic neurons in the presence of the antihelmintic levamisole, an acetylcholine receptor agonist. A reduction in the number of eggs indirectly indicates a possible disruption to the cholinergic system [39]. Exposure to levamisole has been previously shown to cause rapid paralysis in *C. elegans* due to hyper-contraction of body wall muscles [40] and to induce egg-laying due to contraction of vulval muscles in wild-type (WT) animals [41]. Notably, Fe exposure caused reduction in egg-laying, indicating that cholinergic neurons may also be affected by this metal. It is important to note that in numerous experiments, the worms exposed to 0.5 mM Fe showed results analogous to controls. This may be explained by the existence in *C. elegans* of regulatory mechanism for iron intake, which still need to be elucidate. . We intend to study this effect by performing a microarray analysis, to identify genes, such as as ferroportin, transferrin and metathioneins, as well as genes assocaiated with antioxidants, which will guide us in delineating how our exposure protocol might be affecting Fe homeostasis in worms. *C. elegans* express three ferroportin orthologs, FPN1.1, FPN-1.2, and FPN-1.3, but their specific roles in Fe export remain to be fully elucideated.

Taken together, our results show that Fe toxicity may cause severe damage, altering neuronal function, as chracterized by behavioral and

morphological alterations in *C. elegans*. Furthermore, our work showed that the nematode is an invaluable model to study Fe toxicity in order to understand its toxicity. Better understanding of molecular mechanisms of Fe-induced damage is deemed instrumental in developing novel and therapeutic modalities with efficacies exceeding the current standard of Fe poinsoning treatment with deferroxamine. The latter has properties that diminish its clinical utility given its acute [42, 43] and chronic [44] toxicity, limiting the dose that can be safely administered. Furthermore, it has a very short plasma half-life [45].

CONCLUSIONS

Our data suggest that the Fe exposure results in multiple biological defects in *C. elegans*, including reproductive and motor impairment, which may be related to oxidative stress and neuronal damage. Accordingly, additional studies should focus on whether these effects occur at low concentrations of Fe in chronic exposure paradigms and the role of Fe in neurodegenerative diseases.

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CONFLIC OF INTEREST DISCLOSURE

There was no conflict of interest in the preparation of this manuscript.

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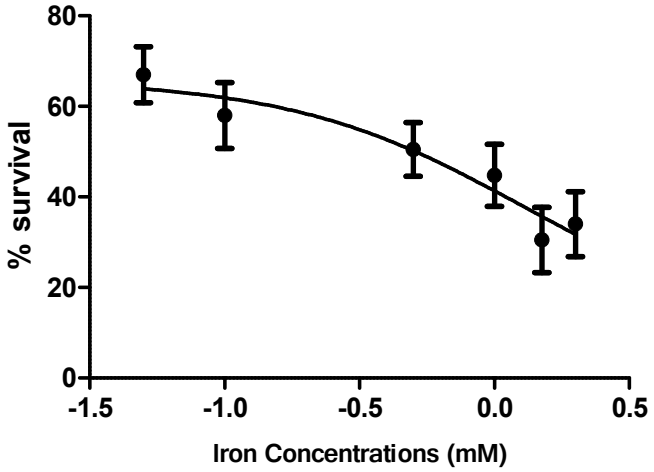

Figure 1: Concentration-dependent response curve for LC50 determination after acute exposure (30 min). The lethality was evaluated by normalizing the data as percentage of control group. Three replicates were performed.

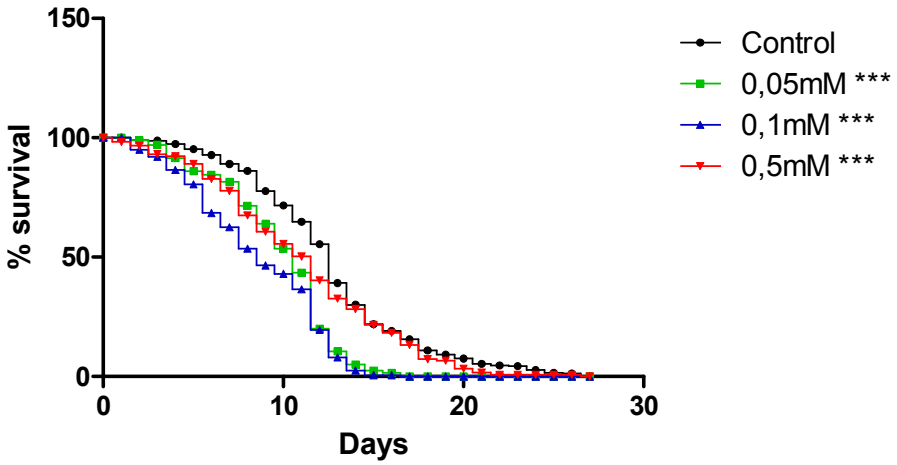

Figure 2: Fe decreases life span in C. elegans. Twenty aged L4 (exposed to Fe when at L1 stage) from each group were transferred to a new NGM/OP50 every day and scored until all animals were dead. All concentrations decreased lifespan compared to control (n=3). *** indicates statistical difference from the control group by one-way ANOVA following by Tukey's post-hoc test ($p<0.0001$).

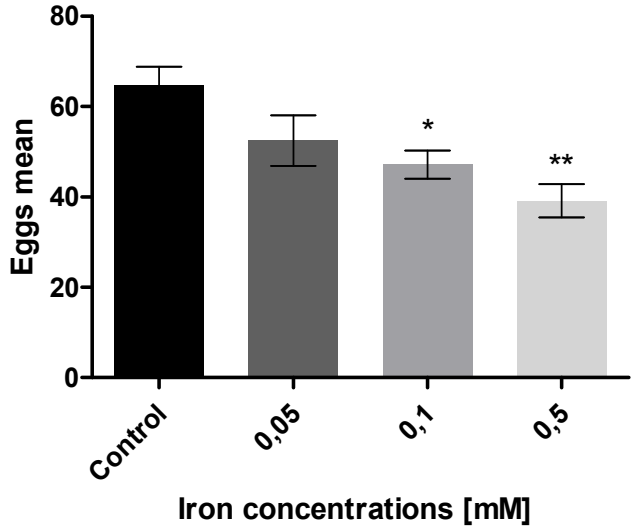

Figure 3: Fe effects on *C. elegans* brood size. The experiments were performed by scoring for four days the eggs layed by individual worms until the end of reproductive period. Each bar represents mean ± S.E.M (n=3). * indicates statistical difference from control group by One way ANOVA followed by Tukey's post-test ($p<0.05$).

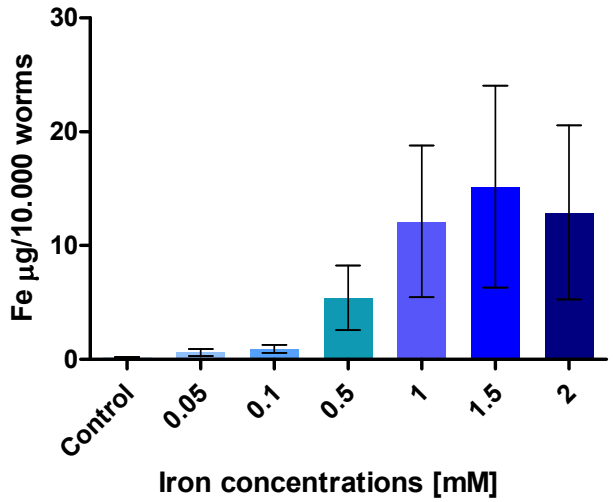

Figure 4: Fe levels in *C. elegans* after acute exposure (30 minutes). The Fe levels were determinate by grafite Graphite Furnace Atomic Absorption Spectrometry (GFAAS). Each bar represents mean (n=3 independent experiments)

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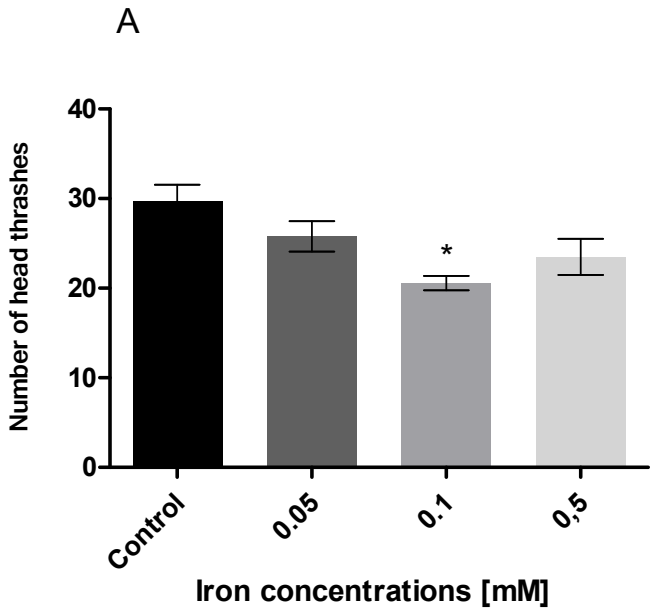

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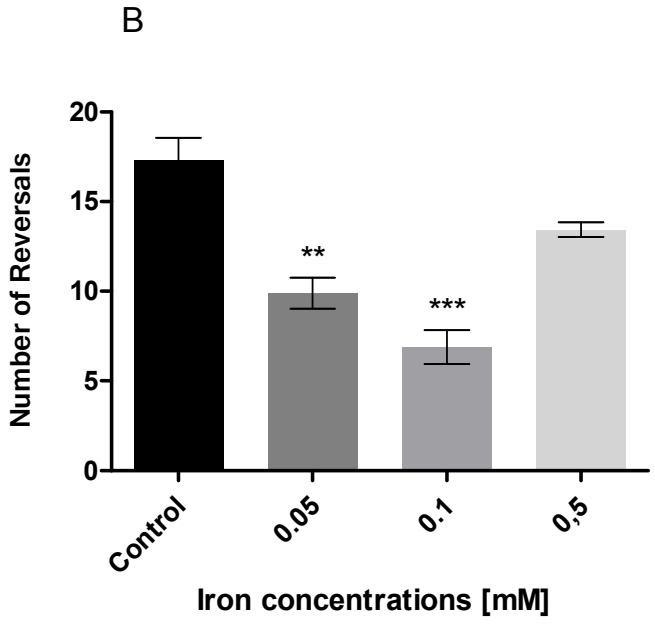

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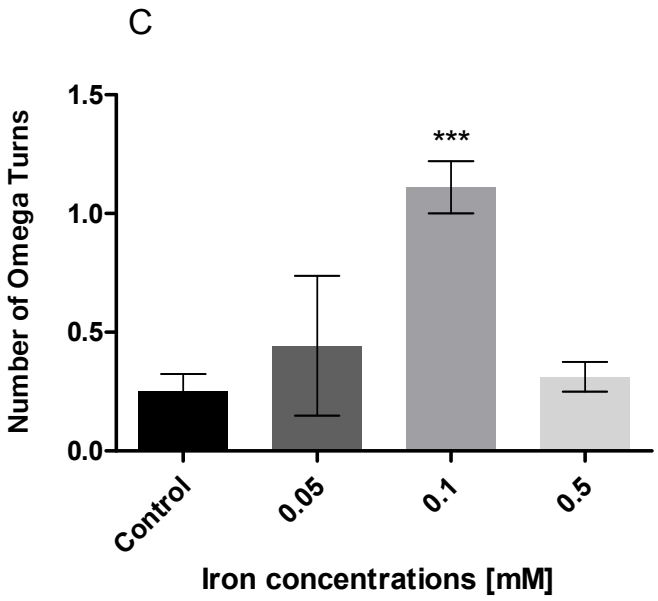


Figure 5: Acute exposure to Fe promotes a decrease in C. elegans basic moviments. A) Head thrash frequency. B) Reverses by nose touch. C) Omega turns by nose touch. Each bar represents mean ± S.E.M (n=5). * indicates statistical difference from control group by one-way ANOVA following by Tukey's post-hoc test ($p<0.05$), ** ($p<0.01$) and *** ($p<0.0001$).

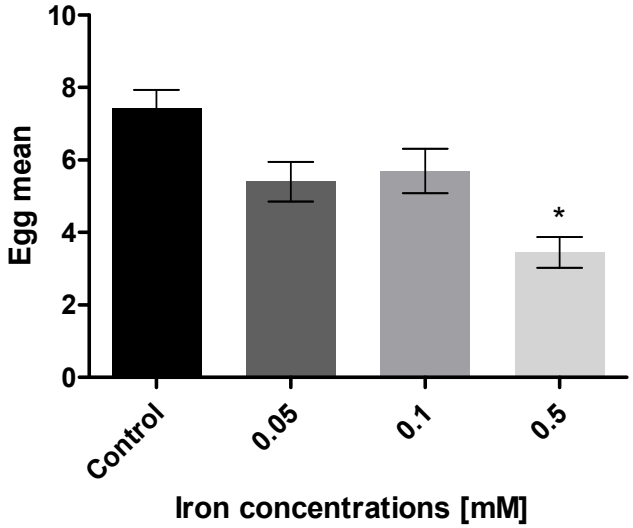
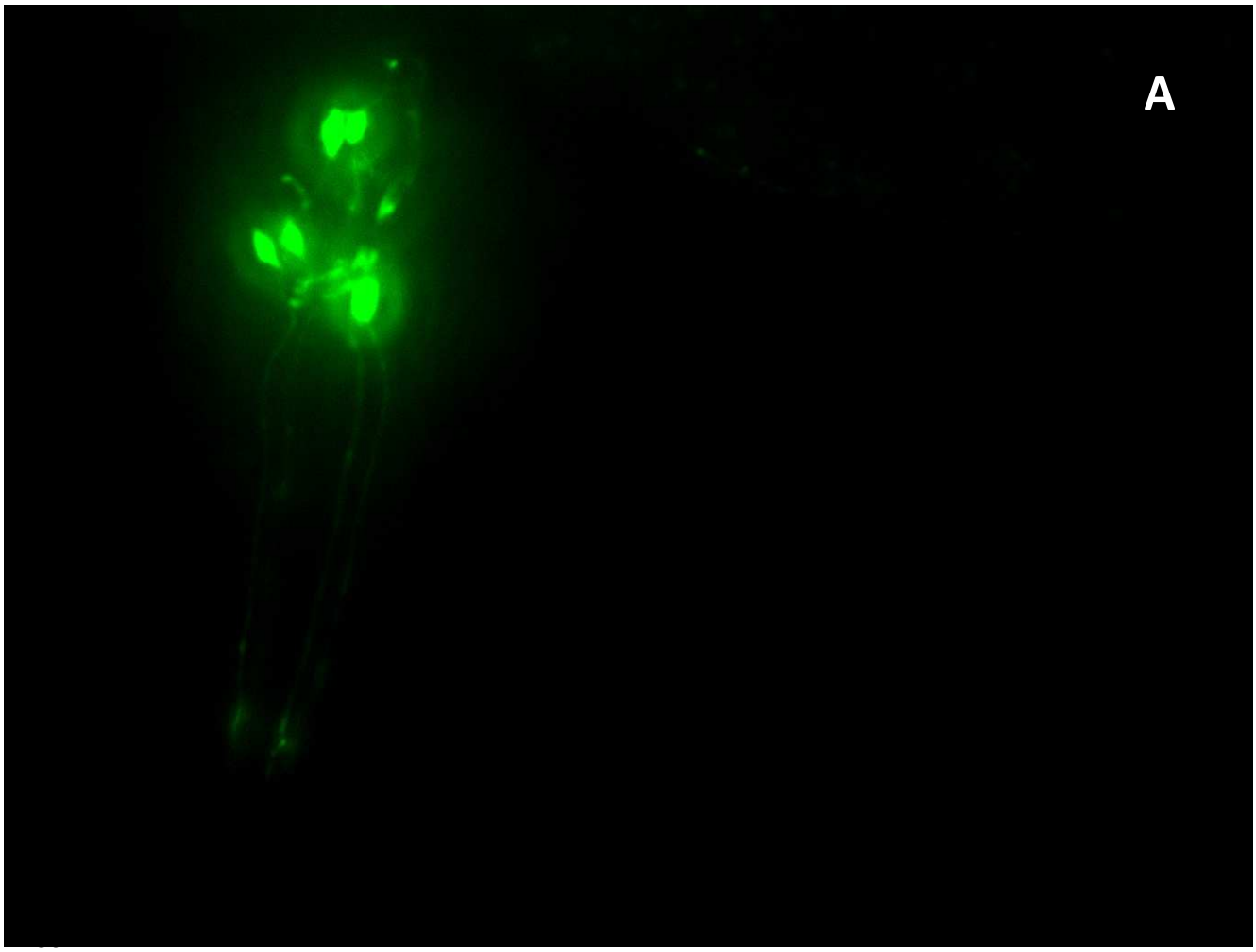


Figure 6: Levamisole induced behavior. When treated with the levamisole, both mock- and exposed adults were observed to hypercontract and lay eggs. Each bar represents mean ± S.E.M (n=3). * indicates statistical difference from control group by One way ANOVA followed by Tukey's post-test ($p<0.05$).



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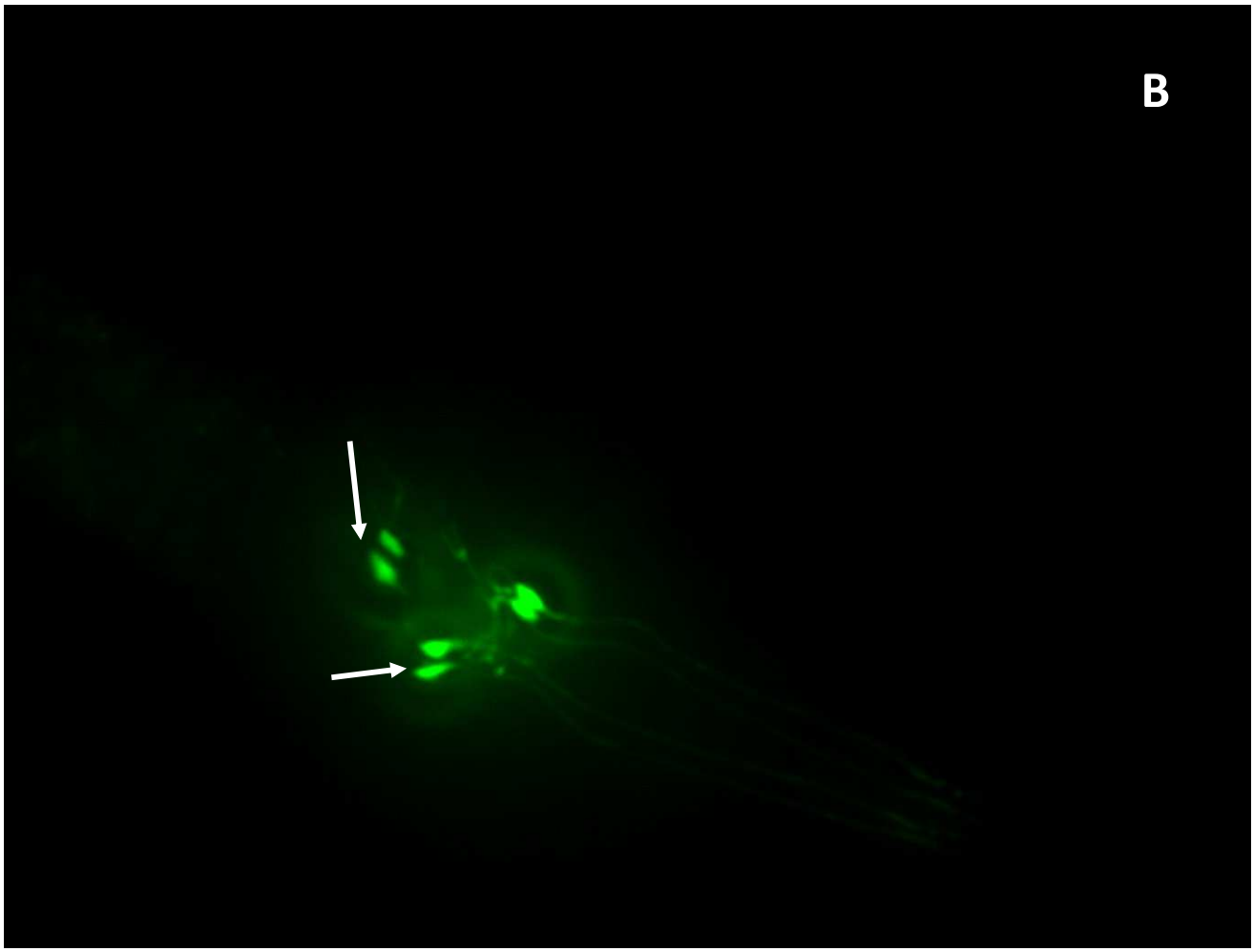

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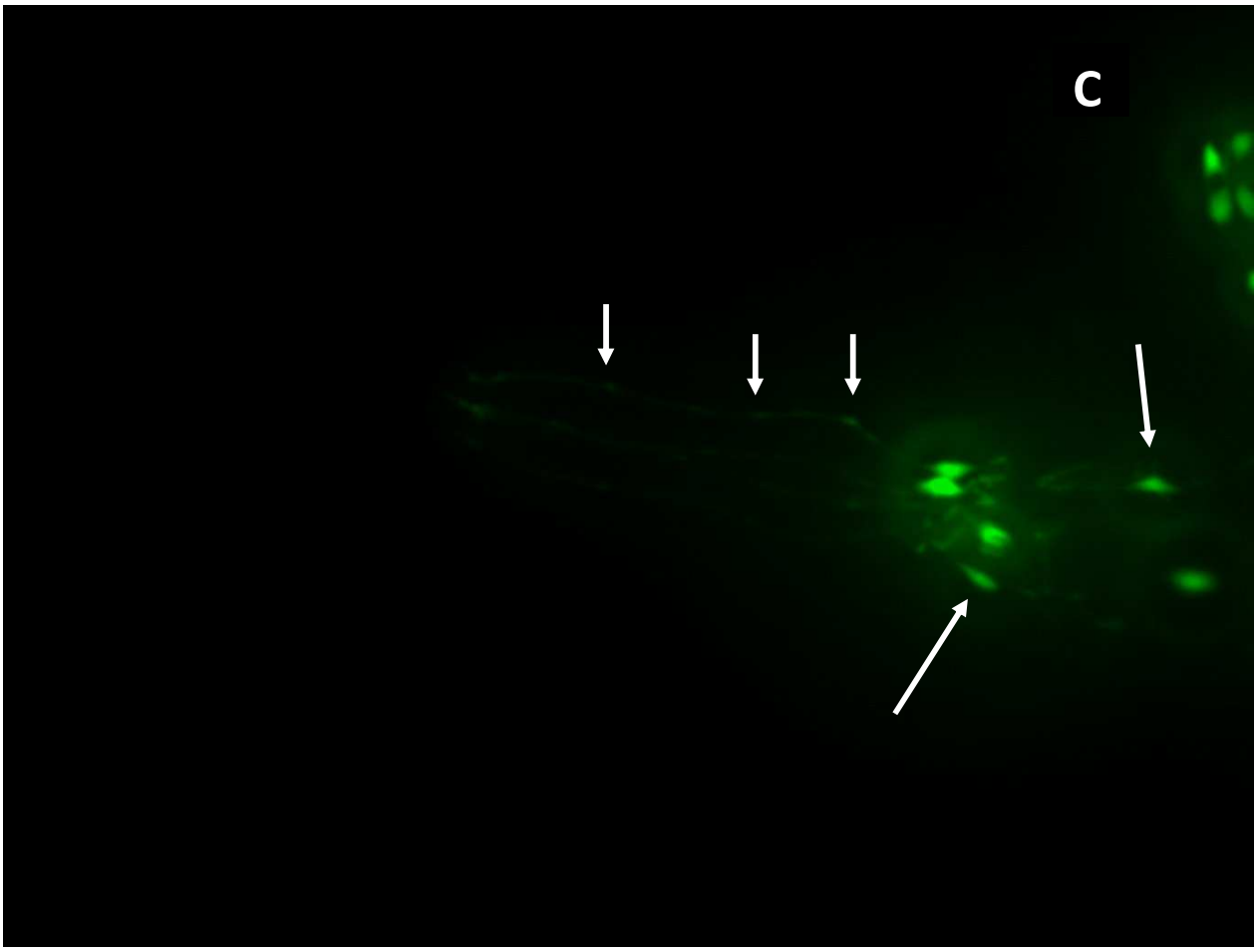

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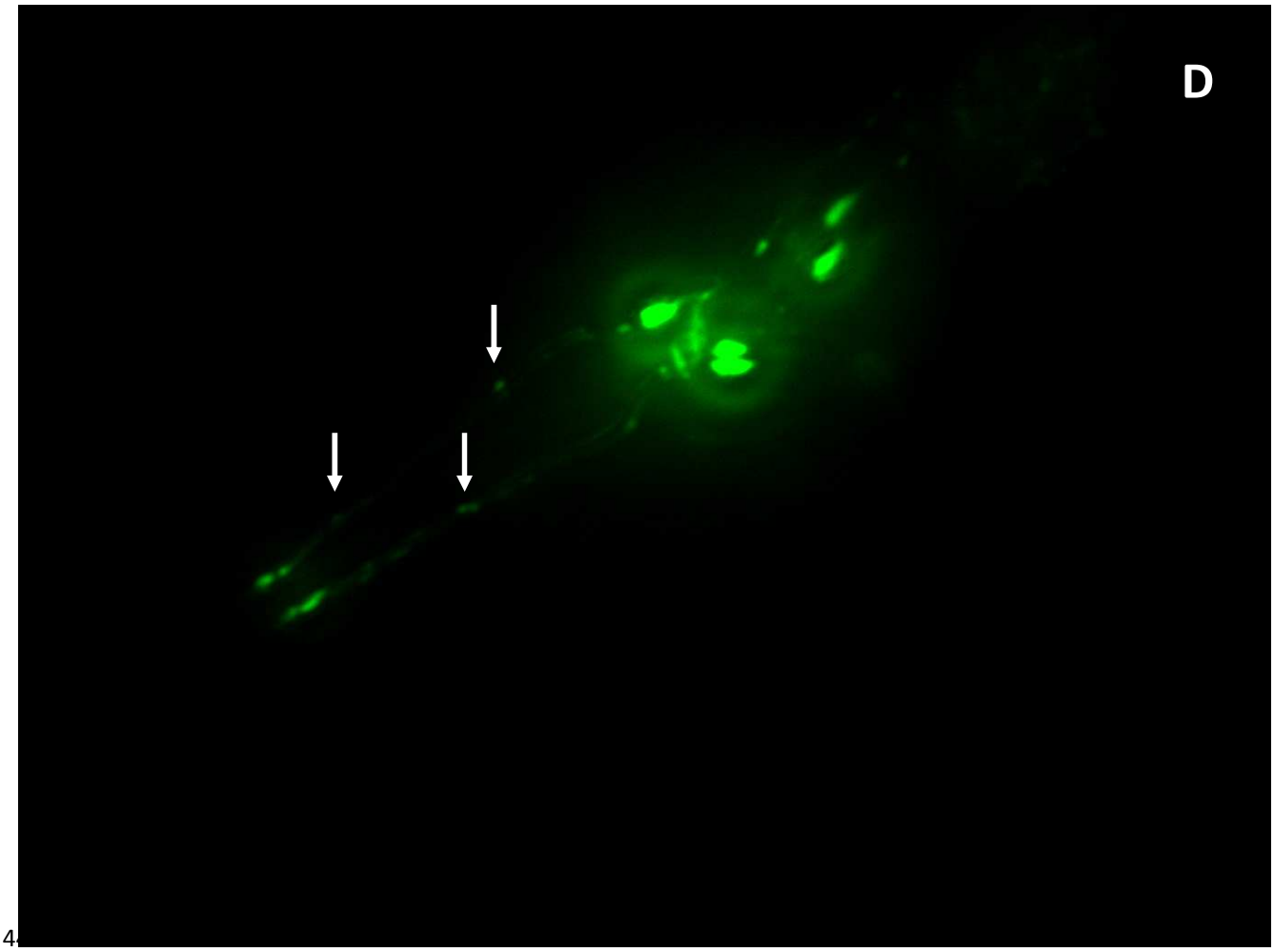

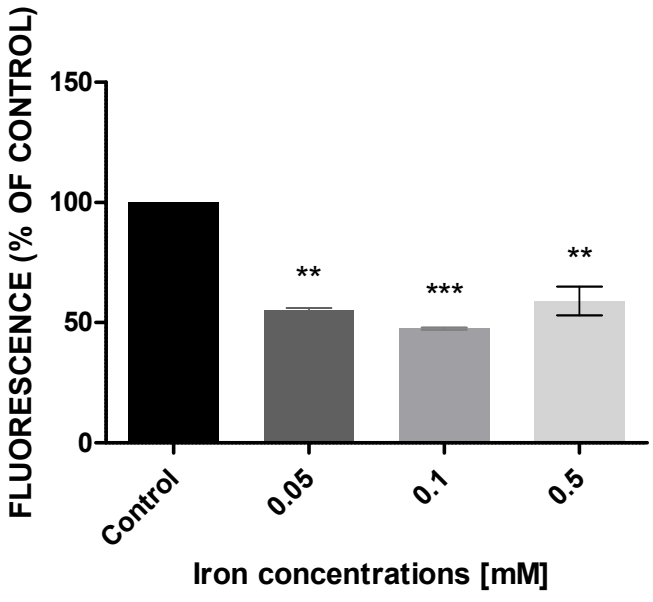

Figure 7: Dopaminergic neurons in alive L4 C. elegans (Pdat-1::GFP) following Fe treatment. Morphological changes were evident with all Fe concentrations and arrows indicate shrunken soma and puncta in the dendrites. (A) Control, (B) 0.05mM, (C) 0.1mM and (D) 0.5 mM. (E) Quantification of the fluorescence, ** indicates statistical difference from control group by one-way ANOVA following by Tukey's post-hoc test ($p<0.01$) and *** ($p<0.0001$).

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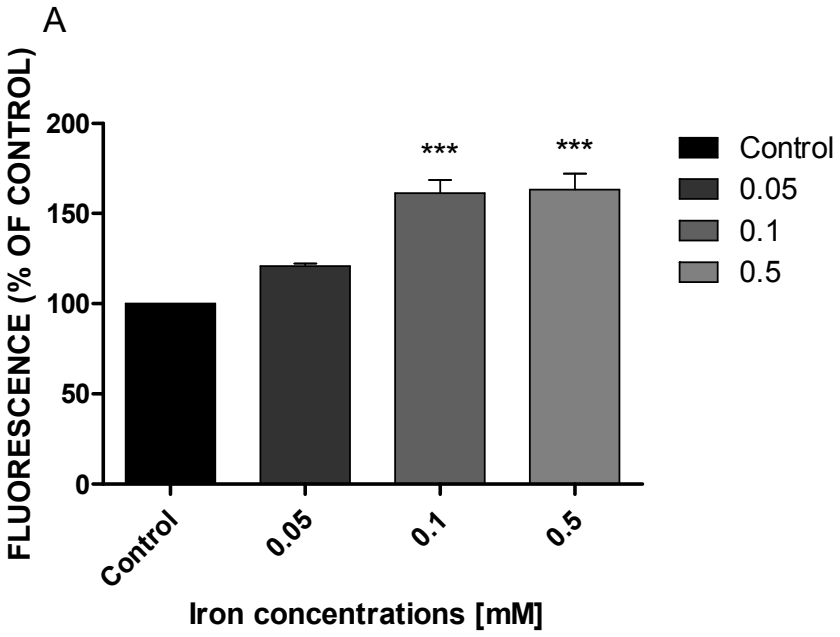

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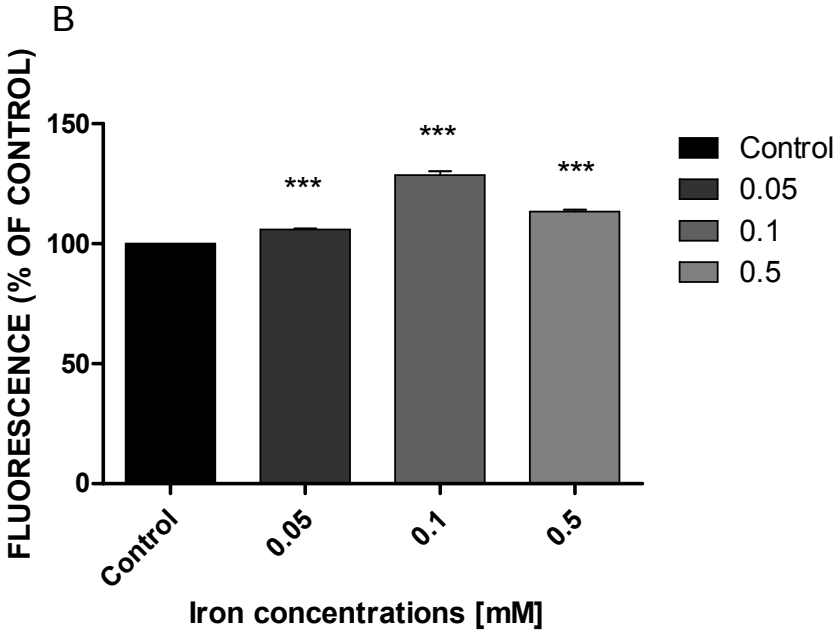

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463 Fig.8: Fe exposure increases ROS production. The lowest doses show a significant
464 increase of ROS levels. (A) L1 worms and (B) L4 worms *** indicates statistical
465 difference from control group by Repeated Measures ANOVA followed by Tukey's
466 post-hoc test ($p<0.0001$)

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