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Measuring “Free” Iron Levels in *Caenorhabditis Elegans* Using Low-Temperature Fe(III) Electron Paramagnetic Resonance Spectroscopy

Kira T. Pate[§], Natalie A. Rangel[§], Brian Fraser[§], Matthew H. S. Clement[‡], and Chandra Srinivasan^{§,*}

[§]Department of Chemistry and Biochemistry, California State University Fullerton, Fullerton, CA 92834

[‡]Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90095-1569

Abstract

Oxidative stress, caused by free radicals within the body, has been associated with the process of aging and many human diseases. As free radicals, in particular superoxide, are difficult to measure, an alternative indirect method for measuring oxidative stress levels has been successfully used in *E. coli* and yeast. This method is based on a proposed connection between elevated superoxide levels and release of iron from solvent exposed [4Fe-4S] enzyme clusters, which eventually leads to an increase in hydroxyl radical production. In past studies using bacteria and yeast, a positive correlation was found between superoxide production or oxidative stress due to superoxide within the organism and EPR (electron paramagnetic resonance) detectable “free” iron levels. In the present study, we have developed a reliable and an efficient method for measuring “free” iron levels in *C. elegans* using low temperature Fe(III) EPR at $g = 4.3$. This method utilizes synchronized worm cultures grown on plates, which are homogenized and treated with desferrioxamine, an Fe(III) chelator, prior to packing the EPR tube. Homogenization was found not to alter “free” iron levels, while desferrioxamine treatment significantly raised these levels, indicating presence of both Fe(II) and Fe(III) in the “free” iron pool. The correlation between free radical levels and the observed “free” iron levels was examined by using heat stress and paraquat treatment. The intensity of the Fe(III) EPR signal and thus, the concentration of the “free” iron pool, varied with the treatments that altered radical levels without changing the total iron levels. This study provides the groundwork needed to uncover the correlation between oxidative stress, “free” iron levels, and longevity in *C. elegans*.

Introduction

The damage caused by oxidative stress in humans has been linked not only to aging [1], but also many diseases, including cancer, heart disease, Alzheimer’s, Parkinson’s, muscular dystrophy, rheumatoid arthritis, diabetes, and cystic fibrosis [2-4]. Oxidative damage is caused by reactive oxygen species (ROS) commonly known as free radicals, including superoxide anions, hydrogen peroxide, hydroxyl radicals, and others [5]. In particular, superoxide anions

*To whom correspondence should be addressed. Department of Chemistry and Biochemistry, California State University, Fullerton, Fullerton, CA 92834, USA. Phone: (714) 278-5439, FAX: (714) 278-5316, chandra@fullerton.edu

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^bIt should be noted that there is no such thing as “free” iron in an intracellular environment and here we use in quotes to indicate the form of iron that is detectable by low-temperature Fe(III) EPR at $g = 4.3$.

(O₂^{•-}), a natural byproduct of cellular respiration, have been found to have deleterious effects on an organism when its levels are high [6]. In yeast and mice, lack of a form of SOD (superoxide dismutase), an antioxidant enzyme that scavenges superoxide, was found to decrease the life expectancy, as well as producing other phenotypes [7-9]. However, the mechanism by which superoxide produces its toxicity is not fully understood.

A current hypothesis regarding the toxicity of superoxide proposes that the superoxide anion oxidizes solvent exposed [4Fe-4S] clusters in certain enzymes, resulting in the inactivation of the enzyme along with the release of “free” iron [10-17]. The released “free” iron can then catalyze, via the Fenton cycle, the formation of highly deleterious hydroxyl radicals (•OH), which can directly oxidize biomolecules, such as DNA [6,18-20]. Therefore, accumulation of superoxide anions leads to oxidative damage on its own and/or by providing the necessary catalyst, iron, for the Fenton reaction. If this hypothesis is true, then oxidative damage due to superoxide anion concentration should be proportional to the observed “free” iron levels.

Past studies have explored the relationship between superoxide buildup and “free” iron levels in unicellular organisms such as *E. coli* and yeast. In both studies, the “free” iron levels in SOD knockout strains that possess higher levels of superoxide were significantly elevated compared to the “free” iron levels in wild-type strain [20,21]. The “free” iron was measured by whole cell Fe(III) EPR (electron paramagnetic resonance) [22]. This spectroscopy utilizes microwave radiation to detect unpaired electrons and can be set at a magnetic field position of $g = 4.3$ in order to measure only high spin, rhombic ferric iron, as ferrous iron signals are too broad to be seen. By measuring at this g -value, the “free” iron pool *in vivo* can be distinguished from other forms of biological iron, such as iron attached to heme proteins or [Fe-S] cluster enzymes, which are EPR detectable at different g -values.

In the present study, we have extended this methodology to a higher multi-cellular organism, *Caenorhabditis elegans* [23]. This 1 mm-long nematode exhibit aging characteristics that are similar to humans and possess high genomic homology (40%) with humans. Even though it was in this organism that single genetic mutations that alter lifespan were first discovered [24] and also antioxidant treatments that alter longevity were screened [25-27] there are not many methods available to measure free radical levels in this system. The method of measuring “free” iron using EPR from the past studies in yeast and bacteria was adapted for this organism with the long-term goals of observing the relationship between superoxide and EPR-detectable “free” iron, observing what variables increase or decrease “free” iron levels, as well as to better understand the mechanism of superoxide toxicity. This study provides the groundwork needed in order to accomplish these long-term goals. We have developed an efficient and effective method of preparing worm EPR samples and we are able to measure “free” iron levels in this organism. We present evidence that the technique is reliable and quantitative in nature. Our data obtained in the presence of heat shock and paraquat treatment indicate that this “free” iron signal intensity can be modulated with conditions that alter free radical levels *in vivo*, thus providing a simple indirect method for monitoring radical levels.

Materials and Methods

Materials

C. elegans wild-type (N2) strain were obtained from the *C. elegans* Genetic Stock Center (Minnesota, USA). The *E. coli* OP50 strain was obtained from Dr. Catherine Clarke's Laboratory (UCLA). *C. elegans* cultivation media supplies were purchased from Fisher Scientific and deferoxamine mesylate (commonly known as desferrioxamine) and methyl viologen (paraquat) were purchased from Sigma. Quartz EPR tubes were purchased from Wilmad Glass (Buena, NJ). Buffers used include: M9 (200 ml 5X M9 Salts, 1 ml 1 M MgSO₄, and 799 ml nanopure water per liter of M9 buffer), 5X M9 Salts (30 g Na₂HPO₄, 15

g KH_2PO_4 , 5 g NH_4Cl , and 2.5 g NaCl per liter of solution), 15% glycerol buffer (15% v/v diluted in M9), and alkaline hypochlorite solution (10 ml nanopure water, 1 ml 5 M NaOH , and 0.8 ml commercial bleach).

C. elegans Cultivation and Synchronization

Worms were grown on plates containing nematode growth medium (NGM) consisting of 8.5 g bacto agar, 1.5 g sodium chloride, 1.25 g peptone, 0.5 ml cholesterol (5 mg/ml stock prepared in 95% ethanol), 0.5 ml 1 M CaCl_2 , 0.5 ml 1 M MgSO_4 , 12.5 ml 1 M KH_2PO_4 and 487.5 ml nanopure water per 500 ml of NGM. The solidified NGM agar plates were spotted with OP50, a strain of *E. coli* used as a food source, and worms were transferred to a new plate by moving an agar chunk containing worms from a stock plate to the new plate. After several days of growth at 22°C, when the worms were predominantly in the gravid (body filled with eggs) stage they were washed off from the plates using sterile M9 solution. The worms were separated from the bacteria using successive centrifugation at 4°C and washing in M9, and then subjected to an alkaline hypochlorite treatment in order to isolate the eggs. The collected eggs were then applied to a new NGM plate containing concentrated OP50 and incubated at 22°C for approximately 48 hours, allowing all the eggs to hatch and grow to the larval stage 4 (L4 stage), thus producing a synchronized population on the plates.

EPR Sample Preparation

The synchronized worms at the L4 stage were then removed from the plates using M9 solution, and the bacteria were removed using centrifugation and repeated washing in M9. The worm count was obtained by diluting a portion of the sample, applying to an unseeded NGM plate, and counting the number of worms under a microscope. The total number of worms in the original sample was then back calculated. This counting procedure was repeated twice for each of the three separate dilutions to determine the number of worms in the original sample. An equal number of worms for each condition (ranging from 12,000 to 25,000) were then transferred by calculated volume to a microcentrifuge tube, which was then centrifuged at 4°C for 3 minutes. Replicate samples for each condition were prepared depending on worm yield. The worm pellet obtained was suspended in 15% glycerol buffer. In experiments where homogenization was performed, samples were homogenized using a handheld tissue grinder at full speed for two minutes. Samples were then treated with desferrioxamine (final concentration 2 mM). In comparison experiments in which desferrioxamine was not added, equivalent volumes of the control vehicle (water) were added. Samples were incubated at room temperature for 15 minutes, then transferred to quartz EPR tubes, which were flash frozen immediately and stored at -20°C until EPR measurements were performed.

Heat Shock and Paraquat (long-term and short-term) Treatment

Samples were prepared as previously stated except that during the last 1.5 - 2 hours of the two-day development of the eggs to the L4 stage, one set of worms was incubated at 35°C, while the other remained at the normal 22°C. After incubation, the standard EPR sample preparation was followed. For long-term paraquat treatment (eggs to L4 stage) samples were prepared as previously stated except that half of the eggs obtained from alkaline hypochlorite treatment were placed on an NGM plate containing 1 mM paraquat, while the other half were placed on a normally prepared NGM plate. Both plates were then incubated at 22°C for two days, followed by the standard EPR sample preparation. For short-term paraquat treatment (1 - 3 hours), synchronized worm populations were obtained as usual by placing all eggs on two regularly prepared NGM plates. After 2-day incubation at 22°C, both plates were washed with M9. Bacteria were removed and concentrated worm pellets were obtained by successive centrifugations. Worms from one plate were then placed on an NGM plate containing 1 mM paraquat, while the worms from the other plate were placed on a normally prepared NGM plate.

Both plates were also spotted with 350 μ l concentrated OP50. After 1 - 3 hours at 22°C, the standard EPR sample preparation was performed.

Low Temperature Fe(III) EPR

EPR measurements were performed using previously published procedures with slight modification [21,22]. Instead of a finger dewar to maintain samples at liquid nitrogen temperature, samples remained near 98 K during the data collection using a variable temperature nitrogen gas setup utilizing a Wilmad quartz dewar insert. EPR spectra were recorded using a Bruker X-band spectrometer (UCLA) at a g -value of 4.3. Parameters used for low-temperature Fe(III) EPR: Center field: 1560 G, sweep width: 500 G, microwave power: 31.8 mWatts, attenuation: 8 dB, modulation amplitude: 20.0 G, modulation frequency: 100 kHz, receiver gain: 2×10^5 , sweep time: 20.97 s, time constant: 81.92 ms, conversion time: 10.24 ms, resolution: 2048 points, number of scans: 8. EPR data processing was done using Bruker WinEPR software and “free” iron quantitation was done by double integrating the signal obtained after baseline correction using the software. Each day, the spectrum of a 22 μ M iron (III) desferrioxamine standard was recorded under identical conditions as that of samples and the double integral value of the standard was compared with the sample to determine their “free” iron concentrations. It should be noted that the “free” iron levels reported here reflects the concentration of “free” iron in a discrete population of worms (in the form of a homogenate) and it is not the concentration on an intraworm basis. In all of the spectra the background signal from the empty cavity was subtracted out. Each sample was often prepared in duplicate or more on a given day and the sample preparation was repeated 2 - 5 independent times. EPR spectra were recreated using SigmaPlot by subtracting the background cavity signal and averaging the spectra for each condition collected on the same day.

Total Fe Measurements using ICP-MS

ICP measurements were carried out using previously published methods [27]. Briefly, worm homogenates were collected in duplicates after EPR measurements and digested in 1 ml of 20% nitric acid (OPTIMA Nitric Acid, Fisher Scientific) at 98°C for 18 h. The digested samples were diluted to 2.7 ml in nanopure water and iron levels were measured using a Hewlett Packard (HP)-4500 ICP/MS at California Institute of Technology (Pasadena, CA). A standard curve was generated using iron standards of known concentration (10 - 200 ppb) and using this curve total iron levels in the unknowns were determined.

Statistical Analysis

All statistical calculations were performed using MINITAB® Release 14 Statistical Software. For any given experiment, samples for both conditions (treated and untreated) were prepared in duplicate (or more) on two or more separate days. Therefore, to determine the statistical difference between conditions, the effects of both the day prepared and the variable treatment were considered. First, a two-way ANOVA was performed to determine the contribution of separate preparation days to the variability among the “free” iron levels. If the separate days did contribute, the variability due to the specific treatment condition was determined based on an F-test from the two-way ANOVA. If the separate days did not contribute, the variability due to the specific treatment condition was determined based on an F-test from a one-way ANOVA.

Results

C. elegans Produce Fe(III) EPR Signal at $g = 4.3$

The first goal of this research was to develop an efficient and reliable method for preparing worm samples in order to verify that wild-type *C. elegans* produce Fe(III) EPR signal at $g =$

4.3 similar to *E. coli* and yeast systems. If so, then secondly, to monitor what causes a change in this EPR measurable “free” iron levels in order to test if this method can provide a simple means to monitor *in vivo* status of oxidative (superoxide) stress levels in the worms. The preliminary Fe(III) EPR data collected for *C. elegans* in our lab was done through preparing mass cultures in liquid medium. While this method consistently produced Fe(III) EPR signal at $g = 4.3$ (data not shown), the procedure was time consuming and a very limited number of samples were prepared in several months time. The method developed and reported here, used worm cultures grown on large plates, which were synchronized and prepared for EPR measurements as described in “Experimental Procedures”. Our initial aim was, therefore, to determine if this protocol was successful in producing a reliable “free” iron signal. Figure 1 shows EPR spectra obtained using three samples, prepared on the same day, under the same conditions using the plate method of culturing. As shown in figure 1, a “free” iron signal at $g = 4.3$ was observed for the worms. Also, the three individual spectra generated using equal number of worms prepared independently, were relatively consistent with one another, indicating that this method of sample preparation yields consistent number of worms per sample.

Homogenization Does Not Significantly Affect “Free” Iron Concentrations

In the preliminary studies, whole worms in described buffers were applied to the EPR tube which often resulted in both a significant number of worms sticking to the sides of the tube as well as pelleting at the bottom of the tube, preventing homogenous sample preparation which is critical for EPR measurements. To refine the methodology, alternate modes of packing the EPR tube were considered and a quick homogenization was tested as a possible solution to these problems. Figure 1 shows that homogenized worm samples yield detectable Fe(III) EPR signal at $g = 4.3$. However, to verify that homogenization does not affect the “free” iron levels within this organism, homogenized samples were compared to non-homogenized whole worm samples (figure 2). As shown in figure 2 the “free” iron signal is not significantly different for these two conditions. “Free” iron quantitation by double integration of replicate samples prepared on the sample day, also shows minimal differences in “free” iron levels for homogenized versus identically prepared non-homogenized samples. This is illustrated in table 1 where no statistical difference (P -value = 0.860) was seen when the comparison was done by preparing samples on three different days using 1 - 3 samples for each day per condition. Our data conclusively prove that a quick homogenization of worms prior to packing EPR sample tubes does not release more iron into this pool indicating that this sample preparation is a viable technique.

The *C. elegans* “Free” Iron Pool Exists in the Form of Fe(II) and Fe(III)

Fe(II) EPR signals are too broad to measure and hence this EPR methodology measures only Fe(III) that is present in this so called “free” iron pool. However, with a simple chemical treatment using desferrioxamine, a cell permeable Fe(III) chelator that chelates both forms of iron by oxidizing Fe(II) to Fe(III), it is possible to see any Fe(II) that is present as part of this “free” iron pool. Past studies done with *E. coli*, showed that the “free” iron was found to be in Fe(II) form as suggested by the lack of detectable signal without the chelator [20]. In contrast, in the yeast study, this iron pool was found to be present only as Fe(III), as the desferrioxamine treatment had no effect on the signal [21]. In order to determine the oxidation state of iron in the “free” iron pool for *C. elegans*, samples were compared with and without desferrioxamine treatment. It should be noted that in studies of *E. coli* and yeast, desferrioxamine was shown not to chelate protein-bound iron, and should, therefore, only chelate the “free” iron [20] under our experimental conditions. As shown in figure 3, the desferrioxamine-treated *C. elegans* samples demonstrate significantly elevated levels of the Fe(III) EPR signal compared to untreated samples, although no change in total iron levels was detected by ICP-MS (data not shown). This EPR experiment was repeated on 4 different days using 1 - 3 independent samples

each day per condition. The “free” iron levels for the two conditions were statistically different (P -value = 0.001) as shown in table 1. This suggests that a portion of the “free” iron present in this pool measured by Fe(III) EPR must exist in the Fe(II) state.

Heat Shock Treatment Decreases the “Free” Iron Pool

Having determined that “free” iron levels are detectable by EPR, the next task was to establish whether or not external stress would alter “free” iron levels. Studies in *E. coli* and yeast have shown that this Fe(III) EPR signal is elevated with *in vivo* superoxide levels, as chemical treatments with radical generators such as paraquat or genetic manipulation (removal of SOD gene(s)) that lead to elevated superoxide levels. In order to uncover the nature of the “free” iron pool in *C. elegans*, heat shock studies were employed to increase free radicals *in vivo*, as maintaining worms at 35°C even for short periods of time has been shown to increase radical production in isolated mitochondria [28,29]. It is also well documented by us and others that during the first few hours of treatment, no deaths are observed in wild-type worms. These results led us to treat the worms at this stressful temperature for 1.5 or 2 hours prior to preparing the samples. As expected, the EPR signal responded to this treatment (P -value for heat shocked vs. control = 0.0016); however instead of seeing an increase in signal intensity that is typically expected due to elevated radical levels, we saw a significant decrease in the “free” iron signal for up to 2 hours of exposure. This experiment was repeated 5 independent times using 1 - 2 samples for each trial and also with varied numbers of worms. All of the heat shocked samples up to 2 hours of treatment consistently produced lower signals compared to the untreated control. A representative comparison is shown in figure 4.

“Free” Iron Pool is Reduced and Total Iron Levels are Unchanged When Eggs are Cultivated on Plates Containing Paraquat

To confirm the decreased “free” iron levels observed by the heat shock induced radical formation *in vivo*, a second study was employed to specifically increase superoxide levels *in vivo* using the herbicide, paraquat [30,31]. In our study we cultivated worms from eggs to larval stage 4 (L4 stage) on plates containing no added paraquat or 1 mM paraquat (for approximately 2 days). EPR samples were prepared and measurements were conducted as previously stated (figure 5, Panels A & B). This treatment also showed a decrease in “free” iron levels similar to the heat shock treatment. This experiment was repeated using 3 - 4 samples prepared on two independent days for each condition and the P -value obtained indicates statistical significance (P -value = 0.019). Although it is interesting to note that treatments that alter free radical levels alter the Fe(III) EPR signal intensity, the trend observed was opposite of what was expected as treatment of yeast with paraquat during growth was shown to increase “free” iron levels. Total iron levels in these samples were measured using ICP-MS after EPR data collection and no difference in the total iron levels was observed (Figure 5, Panel C).

Paraquat Treatment for Shorter Period of Time Increases “Free” Iron Levels

Instead of growing eggs on paraquat for long periods of time until they reached larval stage 4, L4 worms grown normally were treated with paraquat for short periods of time (1 - 3 hours) and samples were prepared and analyzed by EPR. Short-term treatment with paraquat was performed in order to determine if the worms could be captured well before any defensive house-keeping responses could take effect to combat the paraquat induced stress. One hour or three hour treatments did not show any significant differences. However, short-term exposure of the worms to paraquat for 2.5 hours produced an increase in Fe(III) EPR signal intensity ($n = 2$, P -value = 0.048) as shown in figure 6, suggesting that this rise in signal intensity may positively correlate with radical levels *in vivo* prior to any compensatory stress response.

Discussion

Low-temperature EPR-based “free” iron measurements have been a good indicator of superoxide stress (stress due to build-up of superoxide radicals) *in vivo* in yeast and in *E. coli* [20,21]. The method that is utilized to measure “free” iron levels is non-invasive and requires very little manipulation of the organisms. Yeast lacking the antioxidant enzymes Sod1 (CuZn SOD) have high levels of this form of “free” iron and this phenotype can be rescued by the overexpression of human *SOD1* or by providing exogenous Mn(II) in the growth medium [21,32]. Because of the already mentioned advantages of this methodology, in this study we have developed a method for measuring “free” iron levels in a multi-cellular organism, *C. elegans*, by adapting the protocols used in unicellular organisms. One of the key changes we had introduced was homogenization of the worm sample prior to transferring to an EPR tube to obtain a homogeneous sample. This step was not required in the case of unicellular organisms. However, *C. elegans* have a tendency to pellet rapidly and also stick to the walls of the quartz EPR tube during transfer. In order to obtain homogeneous samples and to maintain the quantitative nature of this technique, the homogenization step was required and thus an experiment was required to determine the effects of homogenization. Our data shown in figure 2 and table 1 demonstrate that homogenization did not significantly affect the “free” iron status of the worm samples, and therefore, is a valid step to be incorporated into the proposed method. The oxidation state of the iron in this “free” iron pool measurable by EPR was also examined by treatment with desferrioxamine. Treatment with this Fe(III) chelator for 15 minutes resulted in a significant increase in “free” iron signal (figure 3). Although based on other studies it is known that desferrioxamine treatment for 15 minutes using similar concentrations does not release additional iron into “free” iron pool from iron containing proteins [20,21,33], we carried out fractionation experiments to separate low molecular weight fractions from high molecular weight fractions containing iron. Our study conclusively proved that even up to 2 hour incubation with desferrioxamine does not release iron from high molecular weight iron containing species into low molecular weight iron pool as measured by ICP-MS (data not shown). This conclusively proves that the increase seen with desferrioxamine treatment is not due to additional iron being released into this pool but instead due to portion of the “free” iron pool measured by Fe(III) EPR being present in Fe(II) form. This correlates somewhat with the results of the past study in *E. coli* [20]. It is interesting to note that only yeast “free” iron pool is found fully in ferric form as desferrioxamine treatment in yeast (unlike *E. coli*, rats [34] and worms) does not produce a change in the observed EPR signal. Desferrioxamine treatment for 15 minutes was therefore also incorporated into the methodology, in order to monitor both Fe (II) and Fe(III) forms of the “free” iron present in *C. elegans*. Though treatment with desferrioxamine for up to 60 minutes did not alter the “free” iron levels significantly, 15 minutes was deemed sufficient to chelate this form of iron as monitored by a time course EPR experiment with desferrioxamine (data not shown). We also had to experiment with the number of worms required to see this signal by EPR. We determined that using at least 10,000 worms (L4 growth stage) gave a signal well above the background levels (data not shown). Overall, the method developed and presented here was found to be efficient and reliable in producing a consistent Fe(III) EPR signal at $g = 4.3$ that measures “free” iron levels in *C. elegans* (Fig. 1).

Once we determined that worms also produce this signal at $g = 4.3$, the next step was to explore what variables can increase or decrease this signal using stressors that elevate radical levels. Some of the stressors tested included heat shock and paraquat treatment. Heat shock for brief periods caused the Fe(III) EPR signal to decrease and also paraquat treatment of eggs for 2 days during development produced similar results. Since heat shock and paraquat treatment increase *in vivo* free radical and superoxide levels respectively, we can conclude that the Fe (III) EPR signal intensity changes with amount of free radicals produced. However, the trend observed for *C. elegans* was opposite of what was expected. This is because when a wild-type

strain of yeast was grown in the presence of paraquat, it caused a 5 fold increase in “free” iron levels, similar to the increase seen in SOD knockouts [21]. In *C. elegans*, growth with paraquat or heat stress decreases the signal. One possible explanation for this could be that an adaptive response mechanism is playing a role by up regulating key house-keeping enzymes to help the organism cope with this stress. There is some experimental evidence to support this theory. i) Darr and Fridovich [35] demonstrated that young *C. elegans* can adapt to oxidative stress better than older animals by the up regulation of SODs within 24 hours of exposure to oxygen radical overproducer, plumbagin; ii) paraquat treatment of wild-type *C. elegans* for 1 hour in larval stage 4 increases mRNA levels of SOD1 (a CuZn SOD) and SOD3 (a MnSOD) genes by 2 fold [36]; and iii) our short-term treatment of the worms for 2.5 hours with paraquat elevate the Fe(III) EPR signal, whereas treatment with 1 hour or 3 hours have normal or somewhat lower levels of “free” iron. In any case the fact remains that the signal intensity can be modulated by exogenous stressors that have been characterized to increase radicals *in vivo*. To be certain that the decrease in “free” iron levels seen with paraquat treatment during development was not due to a decrease in total worm iron levels, iron levels were measured using ICP-MS and the data obtained proved conclusively that there is merely a redistribution of the available iron into this pool without the alteration of total iron levels. This suggests that the methodology presented here can now provide a reliable way to further investigate the relationship between cellular oxidative stress produced by various factors with the “free” iron levels and to fully explore what other variables alter this parameter. It will also be interesting to test if cellular iron levels as well as mutations and/or antioxidant treatments that alter longevity have any influence on this EPR measurable “free” iron levels.

Conclusion

A reliable method for preparing Fe(III) EPR samples using *C. elegans* was developed, which successfully produces a “free” iron signal at $g = 4.3$. Homogenization of the worms prior to transfer into the EPR tubes does not significantly affect the “free” iron signal; therefore, it may be used to prepare homogeneous samples. Desferrioxamine treatment increases the “free” iron signal, indicating that both Fe(II) and Fe(III) are present in this iron pool measurable by low-temperature EPR. Treating worm eggs during development with an *in vivo* superoxide generator such as paraquat significantly decreases the “free” iron signal. Although, this is unexpected, there is evidence to support that young *C. elegans* possess the capability to adapt to oxidative stress by increasing their SOD levels. Our data with heat shock, short term and long-term paraquat treatments indicates that this iron EPR signal alters with *in vivo* superoxide or radical levels, which may aid in monitoring oxidative stress status in this model system. This technique may also provide a simple means to monitor oxidative stress changes that occur with age as well as aid in identification of potent antioxidants. Future studies will focus on comparing wild-type and mutants that have altered lifespan to understand the correlation between “free” iron levels and aging.

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Abbreviations used

SOD, superoxide dismutase; EPR, electron paramagnetic resonance; ROS, reactive oxygen species; N2, wild-type; NGM, Nematode Growth Media; PQ, paraquat (methyl viologen).

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Figure 1.
EPR-detectable iron at $g=4.3$ (“free” iron) is seen in young wild-type *C. elegans*. Low temperature Fe(III) EPR spectrum of three individual homogenized samples treated with desferrioxamine (Sample 1 - 3). Each sample contained 12,000 wild-type worms (L4 growth stage) and was collected on the same day. EPR samples were prepared and analyzed as described in the experimental procedures.

Figure 2.

Homogenization of the worms does not alter the “free” iron levels. A representative low temperature Fe(III) EPR spectra of wild-type *C. elegans* with and without homogenization is shown. Samples were prepared on the same day for each condition and the spectra were obtained from samples containing 25,000 worms both treated with desferrioxamine.

Figure 3.

EPR-detectable iron is present in both Fe(II) and Fe(III) oxidation state *in vivo*. A) Low temperature iron EPR spectra of wild-type *C. elegans* with and without desferrioxamine treatment were obtained from homogenized samples containing 12,000 worms each. Three replicates were prepared on the same day for each condition and averaged to obtain representative spectra shown. B) “Free” iron concentration was quantified by obtaining double integration values and calculating the free iron concentration based on comparison with a 22 μ M iron standard. These values represent the average “free” iron levels per worm of three samples for each condition prepared on the same day and homogenized.

Figure 4.

Heat shock treatment at 35°C for 1.5 hours decreases Fe(III) EPR signal at $g = 4.3$. Heat shock treatment was carried out on one part of the sample at 35°C for 1.5 hours while the other part was maintained at normal growth temperature. Spectra were obtained from homogenized samples treated with desferrioxamine containing 24,510 worms each. Samples were prepared on the same day for each condition.

Figure 5.
Paraquat treatment that alters *in vivo* superoxide levels changes EPR-detectable iron levels. Wild type worms were grown from eggs on plates containing no paraquat or 1 mM added paraquat for 48 h until they reached L4 stage. A) Low temperature iron EPR spectra of wild-type *C. elegans* with and without 1 mM paraquat treatment were obtained from homogenized samples treated with desferrioxamine containing 20,000 worms each. Three replicates were prepared on the same day for each condition and averaged to obtain spectra shown. B) "Free" iron Concentration was quantified by obtaining double integration values and calculating the free iron concentration based on comparison with a 22 μ M iron standard. These values represent the average "free" iron levels per worm of three samples for each

condition prepared on the same day, homogenized and treated with desferrioxamine. C) Total iron measurements were performed using ICP-MS on samples prepared from portions of the EPR samples, which were previously homogenized and treated with desferrioxamine. These values represent the average total iron per worm of three different samples prepared in duplicates.

Figure 6.

Wild-type *C. elegans* treated with 1 mM paraquat for 2.5 hours show elevated “free” iron levels. Worms were grown to L4 stage and one sample was then treated with paraquat for 2.5 hours while the other sample was not treated. Samples were prepared as before and spectra were obtained from homogenized samples containing 20,805 worms each and treated with desferrioxamine. Two replicates were prepared on the same day for each condition and averaged to obtain spectra shown.

Table 1

The effect of homogenization and desferrioxamine treatment on the measured “free” iron levels

Effects of Homogenization:				
Day	Homogenized (yes/no)	Number of Samples	Average [Fe] Per Worm * (nM)	Standard Deviation
1	Yes	2	0.209	0.169
	No	2	0.152	0.001
2	Yes	3	0.169	0.160
	No	2	0.095	0.019
3	Yes	1	0.107	-
	No	1	0.132	-
<i>P</i> -value of Homogenized vs. Not-Homogenized = 0.860				
Effects of Desferrioxamine Treatment:				
Day	Desferrioxamine (yes/no)	Number of Samples	Average [Fe] Per Worm * (nM)	Standard Deviation
1	Yes	1	0.154	-
	No	1	0.105	-
2	Yes	2	0.192	0.036
	No	2	0.059	0.049
3	Yes	3	0.130	0.025
	No	2	0.062	0.006
4	Yes	2	0.095	0.019
	No	3	0.044	0.051
<i>P</i> -value of Desferrioxamine vs. No Desferrioxamine = 0.001				

* Average [Fe] per worm here represents the “free” iron concentration