

FLUORIDE INHIBITION OF SUPEROXIDE DISMUTASE (SOD) FROM THE EARTHWORM *EISENIA FETIDA*

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SUMMARY: The occurrence of superoxide dismutase (SOD) in the earthworm *Eisenia fetida* has been demonstrated. The enzyme was inhibited when the worms were exposed to NaF *in vivo* and *in vitro*. Conversely, their exposure to NaF *in vivo* increased tissue glutathione (GSH) levels. Marked inhibition occurred when crude enzyme extract was treated with KCN, suggesting that the enzyme is a CuZnSOD.

Keywords: CuZnSOD inhibition; Earthworm *Eisenia fetida*; Fluoride inhibition of SOD; Glutathione; Superoxide dismutase inhibition.

INTRODUCTION

Under ordinary conditions, most organisms are exposed to different types of endogenous and exogenous toxic substances. Reactive oxygen species (ROS) are a group of such substances, a well-known one being the superoxide free radical ($O_2^{\cdot-}$). There are many biologically generated sources of $O_2^{\cdot-}$ including autoxidations and enzymatic oxidation. A number of enzymes, such as xanthine oxidase,¹ aldehyde oxidase, dihydroorotic dehydrogenase, and several flavin dehydrogenases produce $O_2^{\cdot-}$ during their catalytic cycles.² Organelles such as mitochondria, chloroplasts, microsomes, and nuclei have been shown also to generate this radical.^{3,4} Much of the toxicity of $O_2^{\cdot-}$ is due to the fact that it can act as either a univalent oxidant or a reductant. Superoxide free radical and toxic products of singlet oxygen can both cause deleterious effects on macromolecules such as DNA, RNA, and proteins,^{2,5,6} leading to cell damage and death.

The first line of defense against the action of $O_2^{\cdot-}$ and other ROS is superoxide dismutase (SOD), the enzyme responsible for converting $O_2^{\cdot-}$ into H_2O_2 and O_2 . The resultant H_2O_2 is metabolized to O_2 and H_2O by catalase or glutathione peroxidase (GSH-Px).^{6,7} There is ample evidence that the SODs play an essential role in the biological defense system against oxygen toxicity.² Three types of SOD are currently known: the iron-containing FeSOD, manganese-containing MnSOD, and copper and zinc-containing CuZnSOD.^{2,5}

Because of its role in soil, the earthworm is widely known for its ecological importance. However, information is lacking concerning the effect of fluoride (F^-) on earthworms. Furthermore, little is known about the presence of SOD in the earthworm, or the factors that may affect its activity. In this study we report the occurrence of SOD in the earthworm *Eisenia fetida* and the effects of NaF on its activity and tissue glutathione (GSH) levels.

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MATERIALS AND METHODS

Culture of earthworms: The initial stock of worms was collected from the compost bin of a local gardener. The species of the worm was identified as *Eisenia fetida*, after several specimens were keyed out.⁸ The procedures used for basic care and rearing of the worms were based on American Society for Testing and Materials guidelines.⁹

Dermal contact test: The toxicity test method used was a contact test given by European Economic Community (EEC) guidelines.¹⁰ Earthworms, at least two months old and each weighing about 300–600 mg (wet basis), were used throughout the experiments. The worms were exposed to test material through dermal contact. Prior to exposure, they were placed onto clean moist filter paper for 4 hr to allow fecal matter to be eliminated from their guts. Each worm was then placed individually in a glass vial (7.8 cm long and 2.8 cm in diameter) lined with Whatman No. 3 filter paper wetted with 1.0 mL NaF or de-ionized water as control. The top of the vial was then covered with parafilm ventilated with needle holes. The vials were placed in an environmental chamber at 20±2°C for 24, 48, or 72 hr. Additional 0.5 mL distilled water was added to each vial daily.

Analysis of tissue fluoride: For each treatment group, 24 worms (wet weight: 10–12 g) were dried in a crucible at 80°C for 48 hr. The dried worms were then ground in a mortar and pestle, reweighed, and ashed in a muffle furnace at 600°C for 24 hr. The ashes were then kept in a desiccator until analysis. For F⁻ analysis, about 0.1 g of the sample was placed in a plastic beaker and mixed with 1 mL acetone. After evaporation of the acetone, 20 mL 0.05 M HNO₃ was added. The mixture was stirred for 30 min, followed by the addition of 20 mL 0.1 M KOH and stirring for additional 30 min. Next, 5 mL 0.2 M HNO₃ was added along with 5 mL 0.4 M sodium citrate solution (pH 5.5) containing 1 ppm F⁻. To this mixture 50 mL of TISAB was added. The fluoride content in each sample was then determined using the fluoride ion specific electrode method.

Preparation of crude extract: At the end of the exposure period, five worms were removed and weighed separately. They were then ground for 2.5 min in a mortar containing 1.5 g sterilized silica sand and 5 mL of 65 mM potassium phosphate buffer (pH 7.8). During the grinding an additional 5.0 mL of the buffer was added slowly. The resultant crude extract was filtered through 4 layers of cheesecloth and the mortar and pestle were rinsed with 20 mL of the buffer solution. The combined extract was centrifuged at 8,000 x g for 10 min at 4°C. The supernatant was re-centrifuged at 23,000 x g for 30 min. The supernatant was then placed in a molecular-porous membrane tube (Baxter Spectra/Por, with a 12,000–14,000 molecular weight cutoff), and dialyzed overnight against 26 mM potassium phosphate buffer (pH 7.8) at 5°C. The dialysate was utilized as enzyme.

Enzyme assay: The method for SOD assay was based on the inhibition of nitrite formation from hydroxylamine in the presence of O_2^- generators.¹¹ For this series of experiments, activity units were standardized with the cytochrome c test of McCord and Fridovich,¹ in which one unit of SOD activity is defined as the amount that causes a 50% decrease in the rate of cytochrome c production. For the assay approximately 1/10 SOD activity unit yielded a 50% inhibition of hydroxylammonium chloride. The test protocols were followed exactly except that an amount equal to 0.10 activity unit of xanthine oxidase per mL H_2O was used instead of using the given concentration of xanthine oxidase.

The reaction mixture consisted of 1.0 mL 65 mM potassium phosphate buffer (containing crude enzyme extract), 0.1 mL xanthine solution, 0.1 mL hydroxylammonium chloride, 0.3 mL xanthine oxidase, and 0.5 mL distilled water. The reaction was initiated by addition of xanthine oxidase, and the mixture was incubated in a water-bath at 25°C for 20 min. At the end of the incubation period a 0.5 mL aliquot was removed and added to 0.5 mL sulfanilic acid solution. To this mixture 0.5 mL α -naphthylamine reagent was added, and the mixture was shaken and allowed to stand at room temperature for 20 min. The absorbance of the resulting solution was then determined at 530 nm in a spectrophotometer.

A complete assay consisted of three parts: a blank (H_2O) without either xanthine oxidase or enzyme extract; a complete system minus the enzyme extract (65 mM potassium phosphate buffer was substituted); and the complete system with enzyme extract. The crude enzyme extract was diluted 100 times in 65 mM potassium phosphate buffer. Specific SOD activity was determined by comparison with a standard curve constructed with the use of SOD standards (Sigma Chemical). They consisted of 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 2.0 activity units of the enzyme. The standards were assayed and the SOD concentration was plotted against adjusted percent inhibition.

To test the *in vivo* effect of F^- on SOD in *E. fetida*, the earthworms were exposed to 0, 0.1, 1.0, and 5.0 mM NaF for 24, 48, and 72 hr, respectively. For examining the *in vitro* effect, an aliquot of 0.5 mL 1.0 mM or 5.0 mM NaF, in place of 0.5 mL H_2O , was added to the reaction mixture containing the enzyme extract prepared from the control earthworms.

To find out whether the SOD in the earthworm is a CuZnSOD, 8, 25, and 125 μ M KCN solutions were separately tested for SOD activity. Each of the solutions was added to the reaction mixture containing 0.5 mL H_2O . In this way, the activity of the enzyme extract from the control earthworms was tested in the presence and absence of KCN.

Determination of protein: Protein content of the enzyme extract was determined by the method of Lowry *et al.*¹² The enzyme was usually diluted 50 or 100 times prior to analysis. The amount of protein present in each sample

was determined by comparison with a standard curve constructed by use of protein solutions of known concentrations.

Determination of tissue GSH: To determine tissue glutathione (GSH) levels, earthworms exposed to NaF at 0, 1.0, and 5.0 mM for 48 hr were used. They were ground for 1 min in a mortar containing 0.4 g sterilized silica sand and an extraction medium (3–4 mL/g wet tissue) made up of 10% (w/v) sulfosalicylic acid and 6.3 mM diethylenetriamine pentaacetic acid (DTPA). The mixture was centrifuged at 8,500 x g for 10 min at 4°C. The supernatant was re-centrifuged at 13,000 x g for 20 min. The supernatant was used for GSH determination by the method of Anderson.¹³

Statistics: Both the SPSS statistical package and the statistical functions of Microsoft Excel were used for data analysis. Standard curves were derived using linear regression techniques. To determine any significant differences between treatments, the data was analyzed using analysis of variance (ANOVA). In addition, paired *t* tests were used to compare the SOD activities of extracts treated with KCN. All statistics were performed with α set at 0.05.

RESULTS

Eisenia fetida exposed to 0.1, 1.0, and 5.0 mM NaF for 48 hr showed absorption of increasing levels of F⁻ into the tissue. The increase was manifested in a NaF concentration-dependent manner (Table 1). Significant increases, 29 and 35 times over the control, in tissue F levels were observed in earthworms exposed to 1 mM and 5 mM NaF, respectively.

Table 1. Fluoride body burden of *E. fetida* exposed to NaF

| NaF, mM | F body burden ppm ^a | Percent of control |
|---------|--------------------------------|--------------------|
| 0 | 0.9 ± 0.6 | - |
| 0.1 | 11.5 ± 6.9 | 1278 |
| 1.0 | 26.4 ± 10.4* | 2933 |
| 5.0 | 32.0 ± 12.7* | 3556 |

^aValues are mean ± SD (N=4). *p<0.05.

The effect of NaF at 0.1, 1.0, and 5.0 mM on SOD activity *in vivo* was determined at the end of 24-, 48-, and 72-hr exposure, respectively. Specific enzyme activity decreased significantly with increase in NaF concentration (Figure 1). However, there were no significant differences in the activity between individual concentrations over the three exposure periods. Inhibi-

tion of SOD by NaF was also shown in an *in vitro* study. Under the experimental conditions used, about 70% inhibition occurred when the organism was exposed to 1.0 mM and 5.0 mM NaF (Table 2).

Table 2. Fluoride inhibition of SOD activity *in vitro*

| NaF, mM | SOD specific activity ^a | Percent of control |
|---------|------------------------------------|--------------------|
| 0 | 0.75 ± 0.15 | - |
| 1.0 | 0.24 ± 0.03* | 32 |
| 5.0 | 0.25 ± 0.06* | 33 |

^aValues are mean ± SD (N=3). *p<0.05.

Tissue GSH concentrations were increased in *F. fetida* exposed to NaF. The increases were correlated with increase in NaF concentration up to 1.0 mM, and diminished thereafter (Table 3).

The SOD from *E. fetida* was found to be markedly inhibited by treatment with KCN. As shown in Figure 2, inhibition correlated closely with increasing KCN concentrations. These results suggest that the SOD is a CuZnSOD.

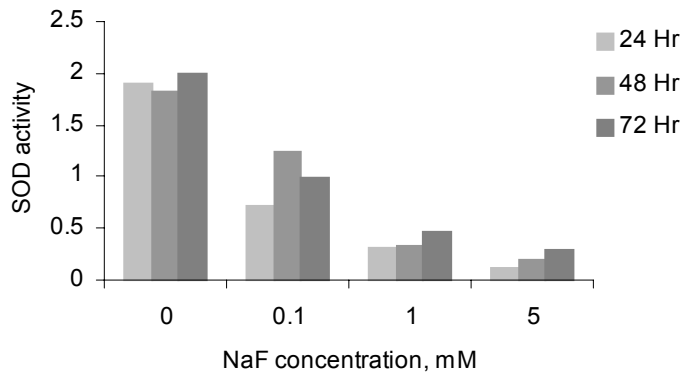
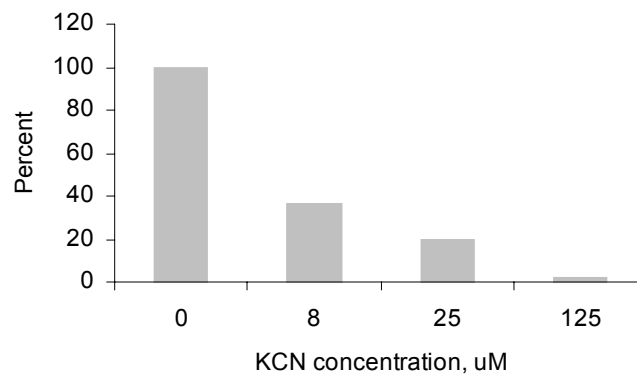


Figure 1. Influence of NaF on SOD activity *in vivo*.

Table 3. Influence of NaF on tissue GSH concentration in *E. fetida*

| Treatment NaF, mM | Tissue GSH $\mu\text{mol/g}$ wet tissue ^a | Percent |
|-------------------|--|---------|
| 0 | 1407 \pm 53 | 100 |
| 0.1 | 1610 \pm 66* | 114 |
| 1.0 | 1801 \pm 94* | 128 |
| 5.0 | 1618 \pm 30* | 115 |

^aValues are mean \pm SD (N=3). *p<0.05.

**Figure 2.** Effect of KCN on SOD activity.

DISCUSSION

Inhibition of SOD and decreases in some antioxidant concentrations by F⁻ exposure have been reported in different organisms including humans.¹⁴⁻¹⁸ Li and Cao observed decreases in the activity of SOD and GSH-Px levels in people living in areas of endemic fluorosis.¹⁴ Similar observations were made by Shivarajashankara *et al.*¹⁸ in 3–10 year-old children with endemic skeletal fluorosis. The fluorotic children exhibited oxidative stress as evidenced by increased lipid peroxidation in their red blood cells. An earlier study by Saralakumari and Rao¹⁹ demonstrated alterations of red cell membranes in humans suffering from F toxicity. In related studies, experimental rats chronically exposed to F in the early stages of life exhibited marked neurodegenerative changes in the brain, suggesting impaired learning and memory, and abnormal behavior patterns.²⁰

In the present study, we demonstrated the occurrence of SOD in the earthworm *Eisenia fetida*. Furthermore, exposure to NaF *in vivo* and *in vitro* significantly inhibited the enzyme (Figure 1; Table 2), supporting earlier reports from use of various organisms. Inhibition of SOD by NaF may be due to a competitive inhibition of the enzyme by the F⁻ ion. The proposed mechanism for F⁻ inhibition of SOD involves its binding to the active site of Cu on SOD, thus displacing water. The affinity of F⁻ for the active site has also been shown to vary with the source of SOD. From their ¹⁹F nuclear magnetic resonance studies, Bianci *et al.*²¹ showed that F⁻ had more than twice the affinity for SOD isolated from yeast than from bovine liver.

The hypothesis of competitive inhibition of SOD by F⁻ also appears to be supported by the results of the *in vitro* study. In this case, *in vitro* exposure to 1.0 and 5.0 mM NaF showed decreases in the pattern of SOD activities that coincided closely with those from the *in vivo* study. These results indicate a similar concentration response pattern in regard to the SOD activity of the earthworms exposed to NaF both *in vivo* and *in vitro*. This would suggest that the binding of F⁻ to the active site of SOD occurs very rapidly *in vitro*, since the extract was exposed to NaF only for a few minutes before the nitrite conversion of the SOD assay was conducted. The results also indicate that the binding of F⁻ to the active site of SOD is not readily, or spontaneously, reversible, and that the reaction rate for F⁻ binding is fairly constant, reaching an equilibrium within a very short period of time. This may explain why the SOD activities of worms treated with identical NaF concentrations appear to be similar for the different exposure periods used in these experiments.

In vivo SOD inhibition by NaF in the present study may be explained by the molecular mechanism of the enzyme and the substrate. The dismutation rate of wild type CuZnSOD is controlled by the diffusional encounter of the reactants.²² That is, reactions occur when the O₂⁻ radical reaches a certain distance from the Cu active site. This reaction is modified by post encounter events, like the passage through a narrow channel of 10 angstrom length from the surface of the enzyme to the active site and the actual transfer of electrons and protons. It appears that the binding to the active site of SOD is also regulated by diffusion rate, thus leading to a greater chance of binding as the concentration of F⁻ increases. This would explain the observed nearly linear response pattern.

The fact that SOD activity was not significantly different between the various exposure time periods, coupled with the similarity of the *in vivo* and *in vitro* results, may be important. It is possible that the binding of F⁻ to SOD, leading to its inhibition, could occur rapidly. This assumption may suggest that, for even a short-term exposure to low levels of F⁻ in the soil, an

immediate and long-lasting effect on the health and functioning of the earthworm could occur.

Total glutathione content refers to the amounts of the reduced form (GSH) and the oxidized form (GSSG) of the substance. According to Anderson,¹³ the normal GSSG concentration in tissue is less than 1% of the total glutathione concentration. Our results showed that the GSH concentration of *E. fetida* tissue was increased by NaF exposure, although no linear relationship was apparent. Our results do not agree with those reported elsewhere.^{14,15,18} It is possible that increases or decreases in tissue GSH concentrations could occur under varying experimental conditions. The increase in GSH concentration may be induced by a decrease in H₂O₂ production as a result of NaF-dependent inhibition of SOD.

It should be mentioned that, while many researchers appear to support the view that fluorosis is closely related to impaired antioxidant systems, a contrasting view is also shared by others. According to Reddy *et al.*,²³ the evidence for an altered oxidative stress and antioxidant balance in fluorosis is inconclusive. From their studies on humans living in an endemic fluorosis area and on rabbits with 150-ppm F⁻ in their drinking water for six months, they reported finding no significant differences in SOD activities, lipid peroxidation, or GSH levels in the blood of human fluorotic patients and F-intoxicated rabbits as compared to respective controls. Chlubek *et al.*,²⁴ in a four-month study of rats with hyperglycemia induced by fluoride in their drinking water, observed a significant decrease in CuZnSOD activity but no changes in glutathione peroxidase activity and malondialdehyde concentration in pancreatic homogenates. They conclude that the hyperglycemia caused by fluoride did not involve activation of free radical production in the pancreas and that inhibition of pancreatic CuZnSOD is probably the result of the direct action of F⁻ on the enzyme.

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