Journal of Exposure Science and Environmental Epidemiology (2014) 24, 180–184

www.nature.com/jes

ORIGINAL ARTICLE

Genetic revelation of hexavalent chromium toxicity using *Caenorhabditis elegans* as a biosensor

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The interaction of heavy metals such as hexavalent chromium, Cr (VI) with the environment drastically influences living organisms leading to an ecological imbalance. *Caenorhabditis elegans,* a saprophytic nematode having 60–80% homology with human genes offers a distinct advantage to be used as a biosensor for the appraisal of heavy metal-induced environmental toxicity and risk monitoring. The present study examines the toxicity effects of $K_2Cr_2O_7$ as Cr (VI) on stress-related gene expression and morphometric parameters of *C. elegans* under *in vitro* conditions to identify genetic markers for environmental pollution. Alterations in growth and modified gene expression were observed in Cr (VI)-exposed N2 worms. The 24-h median lethal concentration for Cr (VI) was observed as 158.5 mgl^{-1}. Use of the responses of stress-related gene expression suggests that *C. elegans* can be used as an efficient biosensor for figuring out the precise route of Cr (VI)-induced environmental toxicity in a quick, simple, and inexpensive manner.

Journal of Exposure Science and Environmental Epidemiology (2014) **24,** 180–184; doi:10.1038/jes.2013.66; published online 23 October 2013

Keywords: *Caenorhabditis elegans*; Cr (VI) toxicity; stress-related gene expression

INTRODUCTION

The global expansion of ecological hazards due to enormous use of heavy metals in various industrial applications has led to a serious environmental threat, generating need for easy, reliable, and efficient detection sources. Toxic metal ions, possibly compete with essential ions (because of similar charge and size) for biological binding sites, leading to structural and functional disturbances.[1,2] Cr (VI) is one such heavy metal contaminant, having potent mutagenic and carcinogenic effects. Cr (VI) is regularly introduced into the environment through several industrial applications viz. electroplating, leather tanning, and pigment manufacturing.[3,4] Exposure to this toxic metal ion not only causes potential human health hazards but also has drastic effects on other life forms. The readability of Cr (VI) to cross the cell permeability barrier because of its resemblance to sulfate ions worsens the problem.[5] Once inside the cellular machinery, Cr (VI) rapidly changes to its unstable transition states viz. Cr (V) and Cr (IV), thereby inducing reactive oxygen species (ROS)-generated oxidative stress in living organisms.[6,7] ROS are chemically active molecules generated during cellular metabolism by various stresses. ROS react with biomolecules and damage physiologically vital macromolecules like DNA, lipids, and proteins leading to numerous age-related diseases like cancer, arthritis, cataract, osteoporosis, type 2 diabetes, hypertension, and Alzheimer's disease.[2] Further, Cr (VI) also causes inhibition of protective processes including DNA repair as well as apoptosis.

Environmental contamination in the biosphere can be successfully assessed through several physical assays such as atomic absorption and inductively coupled plasma-emission spectroscopies. The methods, however, are unable to address the bioavailability of a metal or its movement through the food chain. Therefore, specific endogenous species, or biomonitors that can determine the level of metal pollution, its bioavailability, and the effectiveness of bioremediation strategies[8] are the need of the hour. Several invertebrates viz. gastropods, crustaceans, annelids, molluscs, and sponges have shown their potential as biomonitors for metal pollution.[9–11] The extent of metal pollution is assessed through several factors including the rate of development and survival after exposure and differences in species diversity between contaminated and pristine locations.[12,13] *Caenorhabditis elegans,* a free living soil nematode and an established animal model in ageing studies, is emerging as a popular biomonitor for environmental modeling and toxicity assessment studies. This multicellular, non-parasitic, bacterial feeder nematode has been used in several toxicity studies because of its well-characterized genetic, physiological, molecular, and developmental stages, serving as a living biomonitor of ecological turbulence.[14–16] The short life cycle and lifespan, easy and inexpensive *in vitro* maintenance, limited number of somatic cells (959), completely sequenced genome, and vast occurrence in soil ecosystems are few characteristics of the nematode, which add to its utility as a biomonitor.[17] *C. elegans* offers the advantage of an *in vivo* system that is much simpler than the mammalian system while still sharing 60–80% homology with the mammalian genes.[7] *C. elegans* as a model system affords several advantages viz. small size (1.5 mm), short lifespan (3 weeks), and rapid life cycle (3 days). It is one of the well-characterized nematode species having the completely sequenced genome, well-characterized developmental process, and completely mapped neurons (302) in the nervous system.

Owing to the genetic pliability of *C. elegans* and several transgenic worms containing chimeric reporter genes (regulatory region from a stress inducible gene), it can be easily screened for assessing the environmental stress exposure.[16] Several toxicity end points such as mortality, lifespan, reproduction, and feeding are readily detected and well documented in *C. elegans*.[13] The present study evaluates the toxicity effects of Cr (VI) on the lifespan and survival of *C. elegans* and revealing the toxicity effects

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Received 13 May 2013; accepted 23 July 2013; published online 23 October 2013

of Cr (VI) through transgenic (green fluorescent protein; GFP) worms. The study also identifies the expression of sensitive genes and their quantification through real-time PCR. The study would be beneficial in elucidating the various stress genes and their involved mechanisms serving as a biomarker for assessment of environmental toxicity.

MATERIALS AND METHODS

Organisms

The wild-type *C. elegans* Bristol strain N2 and transgenic strains (CL2070 and CF1553) were used in the present study. The organisms were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP50 at 20 °C using the standard method.[18]

Lethal Toxicity Tests

Potassium dichromate ($K_2Cr_2O_7$, Hi-Media, India) was used as the chemical source for Cr (VI). The test consisted of six metal concentrations (100, 10, 1, 100, 10, and 1 μM) prepared in sterile distilled water and a control. Using a dissecting microscope, 10 young adults were transferred onto 24-well tissue culture plates containing 1 ml of the test solution per well. The worms were exposed to the test solutions for 24 h at 20 °C. Following exposure, the numbers of live and dead worms were determined through visual inspection and by probing the worms with a platinum wire under a dissecting microscope.

Lifespan Assay

Age-synchronized N2 worms were used for lifespan assay. Isolated eggs were allowed to hatch on NGM plates previously spotted with or without different concentrations of Cr (VI) viz. 1, 10, 100, and 1 mM until L4 stage. Thirty to forty L4 moults were then transferred to NGM plates previously spotted with corresponding concentration and 50 μM FUdR (Sigma, St Louis, MO, USA) to block progeny development. Worms were then observed daily for survival and transferred to fresh plates after every 3–4 days to avoid contamination and to assure the presence of the metal throughout the experiment.[19] The experiment was terminated when all the worms were scored as dead or censored. Five independent trials were performed for all the treatments.

Pharyngeal Pumping

To investigate the effect of Cr (VI) concentrations on pharyngeal pumping of the worms, the movement of the pharynx terminal bulb was recorded. Worms were grown on NGM plates, with or without aforesaid Cr (VI) concentration from L1 stage. Pumping records were made for 20-s intervals on adult worms at day0 and day5. The experiment was performed at room temperature and worms were scored on a bacterial lawn using a stereoscopic microscope (Olympus BH2, Japan).[20] The test was performed three times independently.

ROS Detection Assay

Intracellular ROS level was determined using a non-fluorescent dye, dichloro-dihydro-fluorescin-diacetate (H_2DCF-DA, Sigma). Worms were synchronized on NGM plates as described earlier. At day 2 of adulthood, 30 age-synchronized worms were collected in 300 μl of 0.1% phosphate-buffered saline with 0.1% Tween 20 (PBST). Worms were washed three times with PBST and the worm lysate was prepared by an equally timed homogenization and sonication (Branson Sonifier 250; VWR Scientific, Suwanee, GA, USA). The homogenized samples were transferred to 96-well plate and before reading, 15 μl of 10 mM H_2DCF-DA was added to each well. Fluorescent readings were measured in every 20-min interval for 2 h 30 min at 37 °C by using a fluorescent microplate reader (Spectramax M2, Molecular devices). Excitation and emission were read at 485 and 535 nm, respectively.[21]

GFP Visualization and Quantification

The transgenic strains (CL2070 and CF1553) of *C. elegans* having an inducible GFP-reporter-tagged to hsp-16.2::gfp and sod-2::gfp, respectively, were used for intracellular localization of genes. Age-synchronized L4 worms were transferred to NGM plates treated or not with different concentrations of Cr (VI). Worms were then mounted on a 2% agarose pad and visualized under a microscope (Carl Zeiss Axio Scope A1). Photomicrographs for the transgenic *C. elegans* strain CL2070 were taken after exposure of the worms to 37 °C for 2 h and their substantial recovery at 20 °C for 4 h, whereas the photographs for strain CF1553 were taken directly at day3 of the treatment.[22]

RNA Preparation and Quantification of Stress Genes

Following 24-h incubation with exposure to sublethal (1 μM) concentration of Cr (VI), worms were collected for the preparation of RNA. Total RNA was isolated using TRIZOL reagent (Invitrogen). First strand cDNA synthesis was performed with an equal amount of RNA by Thermoscript RT-PCR kit (Invitrogen) as per the kit's instructions. The genes observed through real-time PCR were as follows: *hsp-16.2, sod-3, cat-2,* and *gst-7*. The house-keeping gene *act-1* was used as an internal control. The primers were designed on the basis of the sequences retrieved from the *C. elegans* database (http:// www.wormbase.org), (primer sequences are available on request). mRNA expression was quantified using the SYBR green detection method on an Applied Biosystems 7900 HT fast real-time PCR system. Relative quantification for the expressed genes was done using the comparative C_t ($\Delta\Delta C_t$) method.[23]

Statistical Analysis

Median lethal concentrations (LC_{50}s) were derived through the Probit analysis. The statistical differences between the control and treated worms were determined with the aid of the parametric *t*-test. Significant difference between the lifespan of treated and control worms under both normal and stress conditions was determined using the Kaplan–Meier survival assay in MedCalc software. Data other than lifespan were statistically analyzed using ANOVA in ASSISTAT statistical assistance software. Difference between the data was considered as significant at $P \leq 0.05$.

RESULTS

Lethal Toxicity (LC_{50}) Results

The toxicity of Cr (VI) as $K_2Cr_2O_7$ was studied using LC_{50} derived through Probit analysis (Figure 1). The 24-h LC_{50} of Cr (VI) in *C. elegans* was deduced as 158.50 mg/l (0.7 mM), which indicates the acute toxicity of chromium to living organisms. On the basis of results of the acute toxicity test, four concentrations viz 1, 10, 100, and 1 mM were selected for observing the lifespan and other parameters in *C. elegans*.

Alteration in the Mean Lifespan of Wild-type N2 Worms

To investigate the toxic effects of Cr (VI) as $K_2Cr_2O_7$ on the lifespan of wild-type *C. elegans*, age-synchronized N2 worms were subjected to different concentration of Cr (VI) viz. 1, 10, 100, and 1 mM at 20 °C. Worms were observed daily for survival by touch-provoked method[24] and the survival of treated worms was compared with non-treated worms. It was observed that Cr (VI) at lower concentrations viz. 1 and 10 μM did not affect the lifespan of N2 worms severely but a slight reduction of 1 and 2 days was recorded at the respective concentrations. The mean lifespan of worms was greatly reduced at the other tested concentrations (100 and 1 mM) of $K_2Cr_2O_7$ (Table 1) depicting the drastically lethal toxicity of Cr (VI). A significantly reduced mean and maximum lifespan of 100 and 1 mM Cr (VI) treated worms (Table 1) depicts the acute toxicity of Cr (VI) at higher levels. The χ^2-values for worms treated with 100 and 1 mM concentrations of Cr (VI) was 43.07 and 105.68, respectively, with an associated *P*-value of <0.0001. The survival curves differ significantly and the above concentrations of Cr (VI) have a drastic influence on the lifespan of N2 worms.

Effect on Pharyngeal Pumping

Pharyngeal pumping or the speed of pharynx contraction normally decreases during worms' aging and any alteration in pumping can induce dietary restriction (DR)-like effects affecting the lifespan of worms.[25] To investigate this possibility, the

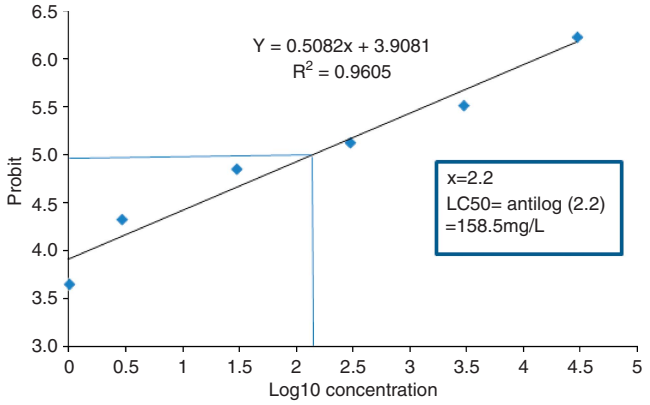

Figure 1. Lethal concentration (LC_{50}) estimation after 24-h exposure to $K_2Cr_2O_7$ in *C. elegans*.

Table 1. Cr (VI)-induced lifespan variation in *C. elegans*.

Treatments	[a]*Mean lifespan*	[a]*Maximum lifespan*	*Log-rank significance*	χ^2
Control	17.14 ± 0.35	28.67 ± 0.33		
1 μM	15.67 ± 0.34	28.33 ± 0.33	$P = 0.0386$	4.2775
10 μM	14.89 ± 0.33	27.67 ± 0.33	$P = 0.0018$	9.7848
100 μM	12.20 ± 0.19	24.67 ± 0.33	$P < 0.0001$	43.0667
1 mM	8.56 ± 0.19	17.33 ± 0.33	$P < 0.0001$	105.6765

[a]Values indicate ± SE.

movement of the pharynx bulb worms treated with Cr (VI) concentrations was recorded for 20 s on adult day0 and day5 (Figure 2a). We did not observe any decrease in pharyngeal pumping on day0 of adulthood, at lower concentrations of Cr (VI), whereas a significantly increased pharyngeal pumping was found in adult day5 worms indicating no DR-like effects because of Cr (VI) concentrations. The worms having a treatment of 1 mM Cr (VI), however, had a reduction in pharyngeal pumping signifying the oxidative damage due to ROS induction at higher Cr (VI) concentrations.

Oxidative Stress Results

The oxidation of H_2DCF to the fluorescent 2′-7′-dichlorofluorescein is a common means of measuring ROS *in vitro*. The fluorescein derivative H_2DCF-DA remains non-fluorescent until the acetate group is removed by intracellular oxidation within the cell. Figure 2b depicts that the ROS levels were increased in all the tested concentrations of Cr (VI). The rate of increase was in direct correlation with the Cr (VI) concentration as the generation of free radicals increased at higher chromium concentrations.

Enhanced Expression of HSP-16.2 and SOD-3 due to Cr (VI) in Transgenic *C. elegans*

The possibility of transgenic *C. elegans* being a biosensor for environmental toxicity monitoring was tested using transgenic lines of heat shock protein *hsp-16.2::gfp*, and superoxide dismutases *sod-3::gfp* were studied. Transgenic nematodes were exposed to sublethal concentration (1 μM) of Cr (VI) for 24 h to evaluate the stress-related molecular responses due to Cr (VI) in *C. elegans*. HSP-16.2 is an antistress reporter protein, having a key role in longevity, whereas superoxide dismutase (*sod-3*) is an antioxidant enzyme, an equally important regulator of lifespan and stress resistance. Cr (VI)-treated worms showed significantly increased fluorescence intensity of HSP-16.2 and SOD-3 protein as compared with that of untreated worms (Figure 3).

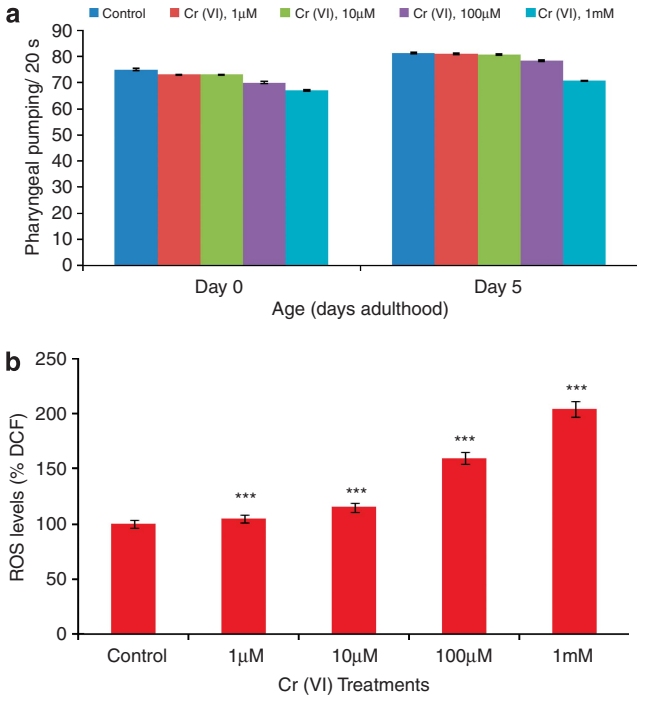

Figure 2. (a) Effect of Cr (VI) concentrations on pharyngeal pumping of N2 worms. (b) Effect of Cr (VI) concentrations on ROS levels in N2 worms. ***Significant *P*-values; statistically significant.

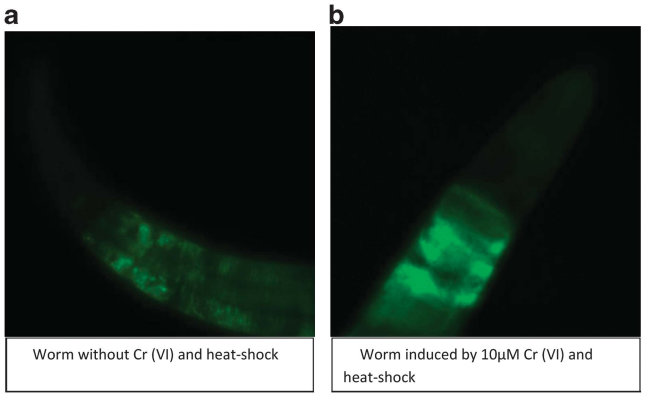

Figure 3. (a and b) Effect of Cr (VI) concentrations on transgenic *C. elegans*.

mRNA Expression of Stress Inducible Genes

The expression levels for various antioxidant genes were quantified using N2 nematodes (Figure 4). DAF-16 is a major transcription factor in *C. elegans* that regulates the lifespan, stress resistance, and antioxidant gene expression through nuclear localization. Quantitative real-time PCR results show that it significantly upregulated the mRNA expression of hsp-16.2 ($P < 0.001$) and sod-3 ($P = 0.003$) but did not affect the expression of *gst-7* ($P > 0.5$). The upregulation of *hsp-16.2* and *sod-3* is in consistence with the result for HSP-16.2::GFP and SOD-3::GFP reporters.

DISCUSSION

Heavy metal abundance in the water bodies as a consequence of superfluous anthropogenic activities leads to human exposure via water, food, and air. The higher permeability of most of these metals poses the risk of several neurotoxic disorders leading to an

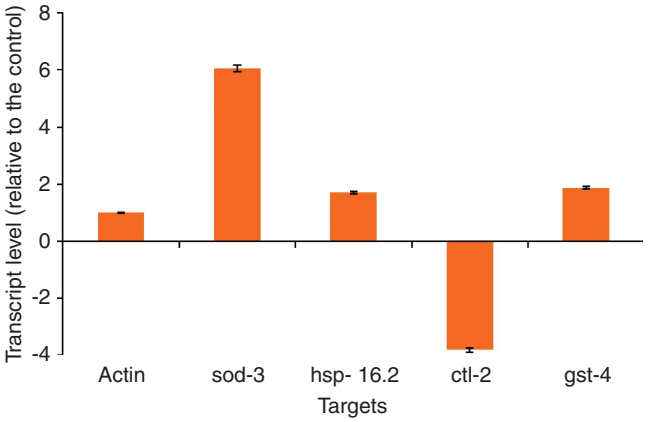

Figure 4. Relative quantification of the stress-induced genes by real-time PCR.

ecological imbalance. Bioassay-based approaches serve as easy, swift, and sensitive screening systems for ecotoxicity monitoring.[26] The success of these approaches, however, requires the fulfilment of two major criteria viz., the omnipresence of bio model and complete knowledge of its genome. Nematodes are the most abundant metazoans present in the soil and aquatic ecosystems serving as popular bioindicators of metal stress because of their ecological significance, short life cycle, and convenience of culturing and *in vitro* maintenance.[27–29] *C. elegans,* a saprophytic soil nematode, is a valuable toxicity model as its results are predictive of outcomes in higher eukaryotes, both at the level of genetic and physiological similarity[8,14,15,16] and at the level of actual toxicity, as many of the basic physiological processes and stress responses that are observed in higher organisms (for example, humans) are conserved in *C. elegans*.[7] The present study evaluates the toxicity effects of Cr (VI) on various patterns of *C. elegans*. Similar to our study, a number of investigators have conducted acute toxicity tests using *C. elegans*.[13,30–32] The 24-h LC_{50} (158.5 mgl^{-1}) value of Cr (VI), as determined in the present study is in accordance with the previously reported toxicity results by Williams and Dusenbery.[27]

C. elegans as a biosensor also enables the detection of organism-level end points (for example, feeding, reproduction, lifespan, and locomotion) and the interaction of a chemical with multiple targets in an organism. In the present experiment, the lethality of Cr (VI) was assessed by observing the mortality of wild-type N2 (Bristol) worms as an organism-level end point for toxicity. The physiological-level alterations due to Cr (VI) were observed as a direct measure of metabolic status in chronic sublethal toxicity assays. Similar to the current examination, a number of studies have emphasized the importance of these end points for chemical-induced toxicity testing in *C.* elegans.[33–36] Heavy metal exposure has been reported to cause a number of irregularities including mitochondrial dysfunction, reduced electron transport chain activity, inhibition of enzyme activity, and indirect ROS generation.

DNA molecules are continuously damaged by environmental stimuli (for example, UV, chemical toxicants, and biological toxins) and endogenous factors formed during oxidative metabolism. At lower metal concentrations, oxidative stress generated because of ROS activates redox-sensitive transcription factors,[37–39] which acts as a foremost factor for Cr (VI)-induced genotoxic and mutagenic effects. Toxic metals such as Cr (VI) possibly interact with thiol groups (in various phosphatases) and oxidize them into disulfide bonds[37] causing protein conformational changes. These protein alterations in turn upregulate various signaling cascades and result in the activation of particular redox-regulated transcription factors.

A simple signal for toxicity can be the measurement of lethality of the nematodes. However, more subtle indicators of toxicity would be desirable. The transgenic *C. elegans* strain that carries a reporter gene (*E. coli lacZ* gene) under the control of a heat shock protein-16 (hsp-16) has been demonstrated as biomonitor for toxicity.[40] Thus, in the present study, the transgenic *C. elegans* strains CL2070 and CF1553, under the control of a hsp-16 and sod-2 stress inducible promoter, respectively, were used. The advantage of using these transgenic *C. elegans* strains is that induction of the hsp-16 can be used as an early signal for intolerability or toxicity to the organism. In the transgenic worms, the stress signal as indicated by β-galactosidase expression in various cells or tissues, can be visualized by histochemical staining and thus have been successfully used by several researchers for environmental toxicity studies.[15,16,38,40–42]

The use of transgenic *C. elegans* in environmental monitoring allows fastidious results with genetically homogeneous animals in a short time. However, for toxicity monitoring, a broad range of stress-related gene promoters for various classes of chemicals needs to be screened.[42] The interactions of various carcinogenic metals with biological components are quite complex. The cellular components in antioxidant defences are vital as they scavenge and balance prooxidants, and ROS, which are attributable to the activities of antioxidant enzymes as well as the action of non-enzymatic antioxidants, thus providing maximal protection for biological sites. The most efficient enzymatic antioxidants comprises catalase, glutathione peroxidase, and superoxide dismutase. Indeed, the capacity to regenerate one antioxidant by another, called an antioxidant network, is driven by redox potentials. By using such sensitive and reproducible detection methods, it is possible to establish significant pollution-induced changes on specific gene expressions and to generate new and accurate assays.

As an adaptive response, animals possess an intrinsic capability of modifying their metabolism and growth according to the changed environmental conditions. These changes are a cumulative result of several modifications in the genetic expression of various genes. Gene expression end points are not only sensitive and useful in estimating the effects of toxicants on expected populations but also may provide insight regarding the mechanisms underlying these effects. In the present study, sensitive genes expressed as part of metal-activated stress response were identified in *C. elegans* using a toxic metal, $K_2Cr_2O_7$ as Cr (VI). Cr (VI) exposure led to increases in the expression of most of the stress-related genes tested, including hsp-16.2, gst-4, sod-1, and ctl-2. A sensitive response at the molecular level may contribute to organism-level resistance, which may be translated into high LC_{50}.

CONCLUSION

The overall results suggest that chromium exhibits a high level of tolerance, which may imply that *C. elegans* possess efficient defense equipment in the form of various antioxidant enzymes that prevents the metal-related damage. It is very clear from the aforesaid account that *C. elegans* complements both *in vitro* and *in vivo* mammalian models in toxicology. The present study with *C. elegans* suggests that this model organism, alongwith the transgenic strain is suitable for studying potential toxicities and stress induction due to heavy metal pollution.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are grateful to Director CIMAP (CSIR), Lucknow, India for providing necessary facilities. Senior author (SKS) is also thankful to Council of Scientific and Industrial Research, Government of India for providing grant-in-aid.

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