Over-Expression of Superoxide Dismutase Ameliorates Cr(VI) Induced Adverse Effects via Modulating Cellular Immune System of Drosophila melanogaster

Prakash Pragya1,3, Arvind Kumar Shukla1,3, Ramesh Chandra Murthy2, Malik Zainul Abdin3, Debapratim Kar Chowdhuri1*

1 Embryotoxicology Section, CSIR-Indian Institute of Toxicology Research, Lucknow, Uttar Pradesh, India, 2 Analytical Section, CSIR-Indian Institute of Toxicology Research, Lucknow, Uttar Pradesh, India, 3 Department of Biotechnology, Jamia Hamdard, New Delhi, India

Abstract

The evolutionarily conserved innate immune system plays critical role for maintaining the health of an organism. However, a number of environmental chemicals including metals are known to exert adverse effects on immune system. The present study assessed the in vivo effect of a major environmental chemical, Cr(VI), on cellular immune response using Drosophila melanogaster and subsequently the protective role of superoxide dismutase (SOD) based on the comparable performance of the tested anti-oxidant enzymes. The immuno-modulatory potential of Cr(VI) was demonstrated by observing a significant reduction in the total hemocyte count along with impaired phagocytic activity in exposed organism. Concurrently, a significant increase in the percentage of Annexin V-FITC positive cells, activation of DEVDase activity, generation of free radical species along with inhibition of anti-oxidant enzyme activities was observed in the hemocytes of exposed organism. In addition, we have shown that ONOO\(^{-}\) is primarily responsible for Cr(VI) induced adverse effects on Drosophila hemocytes along with O\(_2\)\(^{-}\). While generation of O\(_2\)\(^{-}\)/ONOO\(^{-}\) in Cr(VI) exposed Drosophila hemocytes was found to be responsible for the suppression of Drosophila cellular immune response, Cr(VI) induced alteration was significantly reduced by the over-expression of sod in Drosophila hemocytes. Overall, our results suggest that manipulation of one of the anti-oxidant genes, sod, benefits the organism from Cr(VI) induced alteration in cellular immunity. Further, this study demonstrates the applicability of D. melanogaster to examine the possible effects of environmental chemicals on innate immunity which can be extrapolated to higher organisms due to evolutionary conservation of innate immune system between Drosophila and mammals.

Introduction

The ever-increasing human needs have led to countless anthropogenic activities resulting in the release of thousands of chemicals into the environment. The consequence has been detrimental effects of these chemicals on the exposed organism [1]. Since immune system is the first line of defense mechanism in all metazoa, it is likely to be the primary target of environmental chemicals. Considering the importance of this system, there is growing concern to elucidate the effects of such chemicals on the immune system of exposed organism [2]. It has been reported that environmental chemical can impair the immune defense of an organism which eventually leads to chemical induced immunological disorders [3]. Among environmental chemicals, heavy metals have been reported to cause imbalance in the cellular immune homeostasis in exposed organism [4,5].

One of the heavy metals, chromium (Cr), has widespread environmental existence due to its extensive application in diverse industrial processes such as chrome plating, chrome pigmenting, leather tanning, steel manufacturing etc [6]. It exists in several oxidation states in the environment of which Cr(III) and Cr(VI) are the most stable and common forms having biological significance. Cr(III) is a macro-nutrient while Cr(VI), a Class I A human carcinogen [7], is the most toxic form of Cr due to its higher solubility in water, rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids [8]. Earlier studies revealed that Cr(VI) induced reactive oxygen species (ROS) generation causes oxidative stress in the exposed organism which can exert various adverse effects such as apoptosis, modulation of intracellular oxidized states, oxidative deterioration of macro-molecules among others [9,10]. Although limited studies are available to show the immuno-suppressive potential of Cr(VI) in exposed organisms [11–13], those studies were essentially carried out either in vitro or in organisms which have both innate as well as adaptive immunity. Due to the presence of both types of immunity, immune system in those organisms becomes complex and therefore, molecular mechanism underlying the chemical induced modulation of primary innate immune defense remains to be elegantly evaluated.

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* E-mail: dkarchowdhuri@gmail.com
Due to the association of Cr(VI) induced adverse effects with oxidative stress, different anti-oxidants including various modulators of ROS [superoxide dismutase (SOD), catalase] were shown to provide protection against Cr(VI) induced adversities in exposed organism [9,14]. Thus, cytosolic enzyme Cu/Zn SOD, a key enzyme in the dismutation of superoxide radicals [15], assumes significance in the context of Cr(VI) induced adverse biological effects on the innate immune system of exposed organism.

In order to have insight into the mechanism of Cr(VI) induced alteration of cellular immunity and evaluation of possible immunoprotective role of SOD, a suitable model organism with well-characterised genetic network is preferred. In this context, fruit fly *Drosophila*, a well established model organism to study basic principles of innate immunity [16], has been chosen. It relies only on evolutionarily conserved multiple innate immune mechanisms for its defense. The innate immunity in *Drosophila* comprise cellular and humoral responses [17]. Cellular immune response in this organism is mediated by its hemocytes. These cells show extensive homology with vertebrate myeloid lineage especially with mammalian leukocytes [18] eventually protecting the organism from pathogenic infection. In addition, ease of genetic manipulation, limited ethical concern as well as recommendation from European Centre for the Validation of Alternative Methods (ECVAM) for

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**Figure 1. Reduction of total hemocyte count (THC) in Cr(VI) exposed *D. melanogaster* larvae.** Graphical representation of total hemocyte number (%) in Cr(VI) exposed Oregon R larvae after immunostaining by Hemese (H2) antibody (A). Hemocyte population in hml-Gal4 UAS-2xEGFP larvae after Cr(VI) exposure (B). Graph representing total hemocyte count in Cr(VI) exposed hml-Gal4 UAS-2xEGFP larvae as determined by flow cytometry (C). Data represent mean ± SD (n = 3) (20 larvae in each replicate). Significant differences were ascribed as *p<0.05; **p<0.01 and ***p<0.001 as compared to control. Representative confocal microscopic images of the hemocytes in control, 20.0 μg/ml Cr(VI) and 20.0 μg/ml Mo(VI) exposed Oregon R+ larvae for 48 h (D). Extreme right panel represents overlayed images of H2 (green) and DAPI (blue) stained cells. Scale bar: 20 μm.

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**Figure 2. Inhibited phagocytic activity of the hemocytes of Cr(VI) exposed *Drosophila* larvae.** Graph showing inhibition of phagocytic activity (%) in the hemocytes of Cr(VI) exposed Oregon R+ larvae. Bar graphs represent mean ± SD (n = 3) (10 larvae in each replicate). Statistical significance was *p<0.05; **p<0.01 and ***p<0.001 as compared to control.

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toxicological research and testing makes this model useful to study immune response after chemical exposure [19].

The present study, therefore, aims to examine Cr(VI) induced alterations of cellular immune response using Drosophila along with subsequent protection by over-expressing one of the major antioxidant genes, sod, in their hemocytes. Due to evolutionarily conserved innate immune response, the elucidation of Cr(VI) induced alterations in the cellular immunity of Drosophila would add to our understanding regarding innate immunity that can be affected by environmental chemicals in higher organisms.

Materials and Methods

Fly stocks and genetics

Wild type D. melanogaster (Oregon R), Gal4-UAS transgenic lines namely Hemese-Gal4 (He-Gal4), UAS-Sod, UAS-Sod RNi and hML1-Gal4 UAS-2xEGFP (a driven strain having constitutive expression of GFP in hemocytes) were used for the study. Necessary genotypes were generated by standard genetic crosses. The fly strains and their larvae were reared on standard Drosophila food medium (consisting of agar-agar, maize powder, sugar, yeast, nepagin and propionic acid) at 24°C for 30 min. After incubation, cells were washed with PBS and 0.2% trypan blue was added to quench the non-phagocytosed bacteria. The number of hemocytes was normalized in control and Cr(VI) exposed groups and the phagocytic activity was determined according to the number of cells showing phagocytosis in each sample.

Immunofluorescence analysis and hemocyte imaging

Hemocytes were isolated from Drosophila larvae as described previously [18] with minor modifications. Briefly, the hemolymph having hemocyte population was suspended in Schneider’s insect medium (SCM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA) on a coverslip for adherence. The hemocytes were fixed in 4% paraformaldehyde (PFA) washed with PBS, permeabilized in 0.1% Triton-X 100 and then blocked with 0.1% bovine serum albumin (BSA). Immunostaining of hemocytes was carried out by incubating the cells in hemocyte-specific Hemese (H2) antibody (1:100 in 4% BSA) and cleaved caspase-3 antibody (1:200 in 4% BSA; Cell Signaling, Danvers, MA, USA) overnight at 4°C followed by staining with Alexa-Fluor 488 goat anti-mouse (Invitrogen, USA) and Cy-3 conjugated goat anti-rabbit secondary antibodies respectively at 1:200 dilutions in 4% BSA for 2 h. Nuclear staining was performed with 4',6-diamidino-2-phenylindole (DAPI) (1 μg/ml in PBS). For microscopic examination, cells were mounted on a slide using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Immunofluorescence analysis was carried out by capturing the images on a Leica TCS SPE confocal microscope (Nussloch, Germany) and further processed using Adobe Photoshop 7.0 software.

Phagocytosis assay

The phagocytosis assay was carried out essentially by following a previously published method [24] with minor modifications. Briefly larval hemocyte suspension in SCM (supplemented with FBS) was incubated with E. coli-GFP, a Gram negative bacterium E.coli which is tagged with green fluorescent protein (GFP), at 25°C for 30 min. After incubation, cells were washed with PBS and 0.2% trypan blue was added to quench the non-phagocytosed bacteria. The number of hemocytes was normalized in control and Cr(VI) exposed groups and the phagocytic activity was determined according to the number of cells showing phagocytosis in each sample.
Flow cytometric analysis of larval hemocytes, apoptotic cell death and ROS level

Flow cytometry analysis was performed using a Becton Dickinson flowcytometer (BD Biosciences, New Jersey, USA). Ten thousand events were acquired per group and the data were analyzed using Cell Quest software (Mac OS 8.6).

The quantitative estimation of hemocytes isolated from hml-D-gal4 UAS-2xEGFP larvae was carried out. In brief, hemocytes were isolated in PBS and EGFP fluorescence was quantified at an excitation/emission wavelength of 495/519 nm.

For apoptotic cell death analysis, larval hemocytes were stained with Annexin V-FITC essentially following the manufacturer's protocol (Annexin V-FITC apoptosis detection kit). Briefly, cells were suspended in 500 μl of 1X binding buffer (media binding reagent) and subsequently Annexin V-FITC (5 μl) and propidium iodide (PI) (10 μl) were added to the cells. The cells were incubated at 24°C for 10 min in dark. The FITC signal was detected by FL1 (FITC detector) at 518 nm and PI was detected by FL2 (phycoerythrin fluorescence detector) at 620 nm. The log of Annexin V-FITC and PI fluorescence was displayed on the X- and Y-axis of the data report respectively.

The levels of superoxide (O₂⁻) and peroxide (H₂O₂) were measured in larval hemocytes using dihydroethidium (DHE; Invitrogen, USA) and 2', 7'-dichlorofluorescein diacetate (H₂DCFDA) respectively following the methods reported earlier [9,25] with minor modifications. Briefly, isolated hemocytes were incubated with respective dyes at the final concentration of 10 μM for 1 h in dark at 24±1°C. Following a brief washing with PBS, cells were finally re-suspended in PBS for analysis. The fluorescence intensity of the oxidized derivatives of two dyes viz., 2-hydroxyethidium for DHE and 2', 7'-dichlorofluorescein (DCF) for H₂DCFDA was quantified at an excitation/emission wavelength of 535/617 nm and 492/517 nm respectively. The mean fluorescence intensity was used for the estimation of intracellular ROS level in each sample.

Measurement of peroxynitrite (ONOO⁻) generation

Generation of peroxynitrite anion (ONOO⁻) in the cellular system was detected by peroxynitrite mediated oxidation of dihydrorhodamine 123 (DHR 123; Cayman Chemical, USA) to its fluorescent product rhodamine following a published method [26] with minor modifications. Briefly, hemocytes were incubated with 20 μM DHR 123 in PBS for 10 min at 24±1°C and fluorescence intensity of the dye was measured at an excitation/emission wavelength of 500/536 nm on a Varioskan Flash spectrofluorometer (Thermo Fisher Scientific, Finland). The generation of ONOO⁻ was estimated by mean fluorescence intensity of the samples.

Biochemical assays for oxidative stress parameters and DEVDase activity

The absorbance of the coloured products in the following biochemical assays was measured on a Cintra 2 ultraviolet spectrophotometer (GBC Scientific Equipment, Melbourne, Australia).

Different oxidative stress parameters viz., SOD, catalase, thioredoxin reductase (TrxR) enzyme activities, malondialdehyde content and total anti-oxidant capacity were measured in the larval hemocytes.
SOD (superoxide: superoxide oxidoreductase EC 1.15.1.1) enzyme activity in Drosophila hemocyte homogenate was measured following a published method [27] with minor modifications. Briefly, the assay reaction mixture consists of hemocyte sample (Fig. S1), cytochrome C, xanthine and xanthine oxidase in 3 ml potassium phosphate buffer containing EDTA at 25°C. One unit of enzyme activity is defined as the enzyme concentration required for inhibiting the rate of reduction of cytochrome C (optical density at 550 nm) by 50% under assay condition and results were expressed as specific activity in units/mg protein.

Catalase (CAT) (H₂O₂: H₂O₂ oxidoreductase EC 1.11.1.6) activity was measured by following its ability to split H₂O₂ within 1 min of incubation time. The reaction was then stopped by adding dichromate/acetic acid reagent and the remaining H₂O₂ was determined by measuring chromic acetate at 570 nm which is formed by reduction of dichromate/acetic acid in the presence of H₂O₂ as described earlier [20]. The results were expressed as nmol H₂O₂/min/mg protein.

The activity of thioredoxin reductase (TrxR), a substitute for Drosophila glutathione reductase (GR) [29], was measured following a published method [30] with minor modifications. Briefly, to hemocyte homogenate, potassium phosphate and ethylenediaminetetraacetic acid (EDTA) (pH 7.4) were added. After an oxidation step in the presence of reduced nicotinamide adenine dinucleotide (NADPH) and 5,5-dithiobis (2-nitrobenzo-ate) (DTNB), TrxR activity in the sample was assessed at an absorbance of 412 nm. The results were expressed as nmoles NADPH oxidized/min/mg protein using molar extinction coefficient of 13.6 mM⁻¹ cm⁻¹.

The level of malondialdehyde (MDA) as a marker of lipid peroxidation (LPO) was determined based on the reaction with thiobarbituric acid (TBA) as reported earlier [31]. The assay mixture consisted of hemocyte homogenate, distilled water, 10% sodium do-decylsulphate (SDS) and 20% acetic acid solution (pH 3.5). Absorbance was measured at 532 nm against n-butanol and results were expressed as nmoles MDA formed/mg protein.

Protein content in different samples was estimated essentially following a method reported earlier [32] using BSA as the standard.

Total anti-oxidant capacity (TAC) was estimated in the larval hemocytes following the method reported earlier [33] with minor modifications. In brief, hemocyte homogenate was added to diluted 2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) solution at 30°C (Fig. S2). The absorbance was measured at 734 nm exactly 1 min after initial mixing. The percentage inhibition of absorbance at 734 nm was calculated using trolox as an anti-oxidant standard.

DEVDase activity was measured in the larval hemocytes using caspase-3 colorimetric assay kit according to the manufacturer’s protocol. In brief, hemocyte homogenate was mixed with 1X assay buffer and caspase-3 substrate and then the reaction mixture was incubated at 37°C for 1.5 h. Spectrophotometric detection of the chromophore p-Nitroaniline (pNA) at 405 nm was measured for caspase activity which was calculated in terms of μmol pNA released/min/ml of cell lysate.

Determination of SOD activity by in-gel activity assay

The in-gel SOD activity assay was performed essentially following the method described previously [34] with minor modifications based on the reduction of nitroblue tetrazolium (NBT) by O₂⁻ radical into a blue precipitate in the native polyacrylamide gel. Briefly, hemocyte homogenate was loaded on a native polyacrylamide gel and electrophoresed at 4°C for 3 h at 40 mA. Post electrophoresis, the gel was stained with SOD native gel stain comprising NBT, tetramethylethylenediamine (TEMED) and riboflavin in dark. Under fluorescent light, the achromatic bands (clear area) visualized on the gel indicate the SOD enzyme activity present in the sample.

Measurement of the resistance of Drosophila larvae following bacterial infection

The survival of Drosophila larva was measured after they were infected with a Gram negative bacterium, Escherichia carotovora.
carotovora 15 (Ecc15; DSMZ, Germany) as described previously [35] with minor modifications. Briefly, larvae were fed on the mixture of crushed banana and concentrated bacterial pellet in 2:1 ratio for 30 min. The whole mixture was then transferred to a vial containing standard food medium. After 30 min, the larvae were washed in water and transferred individually to an agar plate for scoring at 29°C.

Figure 6. Effect of NAC, L-NAME, SNP on THC and apoptosis in Drosophila hemocytes after Cr(VI) exposure. Graphical representation of total hemocyte number (%) in 20.0 μg/ml of Cr(VI) exposed Oregon R+ larvae along with 10 mg/ml N-acetylcysteine (NAC) or 100 mM N-nitro-L-arginine methyl ester (L-NAME) or 50 μM sodium nitroprusside (SNP) after 24 and 48 h (A1). Representative confocal images of hemocytes from control, 20.0 μg/ml Cr(VI), 20.0 μg/ml Cr(VI) with 10 mg/ml NAC, 20.0 μg/ml Cr(VI) with 100 mM L-NAME and 20.0 μg/ml Cr(VI) with 50 μM SNP exposed Oregon R+ larvae after 48 h (A2). Scale bar: 20 μm. Twenty larvae were taken for each replicate. Graphical representation of percent AV positive hemocytes in Drosophila larvae exposed to 20.0 μg/ml Cr(VI) along with 10 mg/ml NAC or 100 mM L-NAME or 50 μM SNP for 24 and 48 h respectively (B1). Dot plots showing Annexin V-FITC and PI staining in the hemocytes of control (a), 20.0 μg/ml Cr(VI) (b), 20.0 μg/ml Cr(VI) with 10 mg/ml NAC (c), 20.0 μg/ml Cr(VI) with 100 mM L-NAME (d) and 20.0 μg/ml Cr(VI) with 50 μM SNP (e) exposed Oregon R+ larvae after 48 h (B2). Fifty larvae were taken for each replicate. Data represent mean ± SD (n = 3). Significant differences were ascribed as **p<0.01; ***p<0.001 in comparison to control and #p<0.05; ##p<0.01 and ###p<0.001 as compared to 20.0 μg/ml Cr(VI) exposure.

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Cr(VI) Altered Cellular Immunity in Drosophila
Statistical analysis

Prior to analysis, homogeneity of variance and normality assumption concerning the data was tested. Statistical significance of the mean values of different parameters was monitored in control and exposed groups using one way and two way analysis of variance (ANOVA). All the analysis in the same strain with the concentration of metal as a variable was done by one way ANOVA following Dunnett’s test, while comparative analysis between different strains was performed using two way ANOVA followed by Bonferroni’s test for multiple comparisons. Survival analysis was performed using log-rank test. All the statistical analyses were carried out by Prism software (GraphPad version 5.0, San Diego, CA, USA) after setting the level of statistical significance at \( p < 0.05 \).

Results

Detection of metal in the hemocytes of exposed \textit{Drosophila} larvae

A concentration- and time-dependent increase in Cr level was detected in the hemocytes of exposed larvae of different strains (Table S1). A non-significant \( (p > 0.05) \) deviation in Cr levels was observed in the transgenic strains in comparison to that detected in Oregon R\(^+\) larvae. The level of metal in unexposed control larvae was beyond the level of detectable limit.

Reduction in total hemocyte count in Cr(VI) exposed Oregon R\(^+\) larvae

We observed a concentration- and time-dependent reduction in the total hemocyte count in exposed Oregon R\(^+\) larvae with a maximum reduction of \( \sim 56\% \) at 20.0 mg/ml Cr(VI) after 48 h (Fig. 1A, D). Similar to reduced hemocyte number, a significant down-regulation in the gene expression level of \textit{hemese} was also observed in exposed organism (Fig. S3A). Further, reduction in total hemocyte count observed in the exposed \textit{hml\(^+\)-Gal4 UAS-2xEGFP} larvae (Fig. 1B–C) was found to be comparable with Oregon R\(^+\).

Inhibition of phagocytic activity in the hemocytes of Cr(VI) exposed organism

Earlier, it has been stated that the phagocytic activity of a cell can be attributed as an immunological biomarker [36] to demonstrate the potential of a chemical for immuno-modulation. In this context, we observed a significant inhibition of phagocytic activity in the hemocytes of Cr(VI) exposed organism (Fig. 2) with a maximum inhibition of \( \sim 53\% \) at 20.0 mg/ml after 48 h.

Increased apoptotic cell death in the hemocytes of Cr(VI) exposed organism

Since apoptosis can be the predominant mode of cell loss due to hexavalent Cr and not due to hexavalent form of another metal, was further confirmed by exposing the larvae to another metal Mo(VI). Unlike the above, we observed non-significant difference in the total hemocyte count in 20.0 mg/ml Mo(VI) exposed Oregon R\(^+\) larvae for 48 h in comparison to unexposed control (\( \sim 7\% \) reduction; Fig. 1D).

Activation of DEVDase activity in the hemocytes of exposed Oregon R\(^+\) larvae

Similar to the detectable increase in AV positive hemocyte population in Cr(VI) exposed Oregon R\(^+\) larvae at higher concentrations of the test chemical, we also observed an increase...
in DEVDase (caspase 3-like) activity in the hemocytes of exposed organism. Maximum increase in DEVDase activity (~360%) was observed in the hemocytes of larvae that were exposed to 20.0 μg/ml Cr(VI) for 48 h (Fig. 4A). Parallel to the DEVDase activity, immunofluorescence analysis using cleaved caspase-3 antibody also revealed activation of caspase-3 in the hemocytes of exposed organism (Fig. 4B).

Enhanced generation of $O_2^{-}$ in the hemocytes of Cr(VI) exposed Oregon R' larvae

Concomitant with our observations on Cr(VI) induced reduction in total hemocyte count and apoptotic cell death, a significant increase in DHE and DCF fluorescence intensities in the hemocytes of Cr(VI) exposed Oregon R' larvae with a maximum increase in DHE (~302%; Fig. 5A) and DCF (~267%; Fig. 5B) fluorescence at 20.0 μg/ml Cr(VI) after 48 h was observed as compared to control. Further, involvement of $H_2O_2$/OH was resolved by inhibiting the generation of OH radical in the hemocytes of 20.0 μg/ml Cr(VI) exposed *Drosophila* larvae after 24 and 48 h using N-acetylcysteine (NAC), which is reported to scavenge cellular $H_2O_2$ and OH radical [38]. We did not observe any significant beneficial effect on the total hemocyte count and apoptotic cell death of the hemocytes in larvae that were exposed to 10 mg/ml NAC and 20.0 μg/ml Cr(VI) together as compared to that observed with 20.0 μg/ml Cr(VI)-alone exposed organism (Fig. 6A–B; Fig. S4A). Non-significant alterations in the above parameters were observed after exposure of NAC alone to *Drosophila* larvae (data not shown).

Peroxy nitrite generation in the hemocytes of Cr(VI) exposed organism

A concentration- and time-dependent increase in DHR fluorescence was observed in the hemocytes of exposed organism with a maximum increase of ~244% after 20.0 μg/ml Cr(VI) exposure for 48 h (Fig. 5C). On the other, inhibition of ONOO$^-$ generation in the hemocytes of *Drosophila* larvae was achieved by exposing the organism to 100 mM N-nitro-L-arginine methyl ester (L-NAME; a nitric oxide synthase (NOS) inhibitor) [39] (Fig. S4B), with Cr(VI) for 24 and 48 h. A significant increase in the total hemocyte count as well as decreased apoptotic cell death was observed in co-exposed larvae in comparison to that observed in 20.0 μg/ml Cr(VI)-alone exposed organism (Fig. 6A–B). When the larvae were exposed to 50 μM sodium nitroprusside (SNP; a potent nitric oxide (NO) generator) [40] (Fig. S4C) along with 20.0 μg/ml Cr(VI) for 24 and 48 h, we observed a significant reduction in the total hemocyte count and increased apoptotic cell death in the co-exposed group as compared to Cr(VI)-alone exposed group (Fig. 6A–B). However, exposure of L-NAME or SNP alone to *Drosophila* larvae non-significantly altered the above measured endpoints (data not shown).

Generation of oxidative stress in the hemocytes of Cr(VI) exposed organism

We observed a concentration- and time-dependent significant decrease in SOD activity in the hemocytes of Cr(VI) exposed larvae was observed [~37% after 20.0 μg/ml Cr(VI) exposure for 48 h] (Fig. 7A). This observation was further confirmed by in-gel SOD activity assay which showed a trend similar to that observed by the biochemical assay (Fig. 7B). However, unlike SOD, we observed a significant decrease in CAT and TrxR activities only in the hemocytes of 20.0 μg/ml Cr(VI) exposed larvae (Fig. 7C–D). Concomitant with an inhibition of all the above tested enzyme activities, we observed a concentration- and time-dependent significant increase in MDA content in the hemocytes of exposed Oregon R' larvae (Fig. 7E). Further, total anti-oxidant capacity (TAC) declined in a concentration- and time-dependent manner in the hemocytes of Cr(VI) exposed larvae with a maximum ~45% decrease when the organism was exposed to 20.0 μg/ml Cr(VI) for 48 h (Fig. 7F).

Decreased resistance of Cr(VI) exposed Drosophila after Ecc15 infection

We observed a concentration- and time-dependent decrease in the resistance (in terms of survival of larvae) of Cr(VI) exposed Oregon R' larvae infected with Ecc15 in comparison to Ecc15 only infected organism [~48% survival in 20.0 μg/ml Cr(VI) exposed larvae for 48 h following Ecc15 pathogenic infection] (Fig. 8). Before the resistance assay, bacterial load in infected larvae was ascertained by quantifying the bacterial level after natural infection (Fig. S5A).

Immuno-protective effect of sod against Cr(VI) induced alteration in cellular immunity

We observed a significant increase in the total hemocyte count in Cr(VI) exposed *He-Gal4 > UAS-Sod* larvae (~29% higher cell count after 20.0 μg/ml Cr(VI) exposure for 48 h) (Fig. S9A–B; Fig. S3B) and less inhibition of phagocytic activity (~23%) (Fig. 9B) as compared to the respective *He-Gal4* larvae. Further, significantly lesser number of AV positive cells were observed in exposed
He-Gal4 larvae (18%) as against that observed in He-Gal4 larvae (32%) under similar experimental condition (Fig. 9C1–2). Concurrently, we observed 149% decreased DEVDase activity in the hemocytes of exposed sod over-expressing strain as compared to the respective He-Gal4 larvae (Fig. 9D1–2). A similar trend for ROS (O$_2^•$- and H$_2$O$_2$) (Fig. 9E1–2) and peroxynitrite (Fig. 9E3) generation, SOD and CAT (Fig. 9F1–2) activities, MDA content (Fig. 9F3), TrxR activity (Fig. 9F4) and TAC level (Fig. 9F5) was observed in the hemocytes isolated from the exposed larvae of sod over-expressing strain. Further, increased resistance of Cr(VI) exposed He-Gal4 larvae was observed in comparison to the respective He-Gal4 larvae after Ecc15 infection (Fig. 9G; Fig. S5B). When sod was genetically knocked down in the hemocytes of Drosophila (He-Gal4 UAS-Sod RNAi larvae) for 48 h following Ecc15 infection (Fig. 9G), the survival (%) of 20.0 μg/ml Cr(VI) exposed Drosophila larvae (He-Gal4, UAS-Sod, He-Gal4 UAS-Sod RNAi and He-Gal4 UAS-Sod RNAi larvae) was observed to be significantly decreased (Fig. 9G).

Figure 9. Protective effect of sod over-expression on Drosophila cellular immune response after Cr(VI) exposure. Comparative hemocyte population in He-Gal4, UAS-Sod, He-Gal4 UAS-Sod, UAS-Sod RNAi and He-Gal4 UAS-Sod RNAi larvae after 48 h Cr(VI) exposure (A1). Representative microscopic images of hemocytes from control and 20.0 μg/ml Cr(VI) exposed larvae for 48 h (A2). Phagocytic activity (%) of hemocytes of He-Gal4, UAS-Sod, He-Gal4 UAS-Sod, UAS-Sod RNAi and He-Gal4 UAS-Sod RNAi larvae after 48 h Cr(VI) exposure (B). Percent AV positive hemocytes in 48 h Cr(VI) exposed He-Gal4, UAS-Sod, He-Gal4 UAS-Sod, UAS-Sod RNAi and He-Gal4 UAS-Sod RNAi larvae (C1). Representative dot plots for Annexin V-FITC and PI staining in the hemocytes from He-Gal4 control (a) and 20.0 μg/ml Cr(VI) exposed He-Gal4 (b), UAS-Sod (c), He-Gal4 UAS-Sod (d), UAS-Sod RNAi (e) and He-Gal4 UAS-Sod RNAi (f) larvae for 48 h (C2). Comparative DEVDase activity in the hemocytes of He-Gal4, UAS-Sod, He-Gal4 UAS-Sod, UAS-Sod RNAi and He-Gal4 UAS-Sod RNAi larvae exposed to Cr(VI) for 48 h (D1). Representative confocal images of hemocytes from control and 48 h, 20.0 μg/ml Cr(VI) exposed larvae (D2). Comparative levels of O$_2^•$ (E1), H$_2$O$_2$ (E2) and ONOO$^•$ (E3) generation in the hemocytes of 48 h Cr(VI) exposed He-Gal4, UAS-Sod, He-Gal4 UAS-Sod, UAS-Sod RNAi and He-Gal4 UAS-Sod RNAi larvae. Graphical representation of SOD activity (F1), CAT activity (F2), MDA content (F3), TrxR activity (F4) and TAC (F5) in the hemocytes of He-Gal4, UAS-Sod, He-Gal4 UAS-Sod, UAS-Sod RNAi and He-Gal4 UAS-Sod RNAi larvae that were exposed to Cr(VI) for 48 h. Representation of the survival (%) of 20.0 μg/ml Cr(VI) exposed Drosophila larvae (He-Gal4, UAS-Sod, He-Gal4 UAS-Sod, UAS-Sod RNAi and He-Gal4 UAS-Sod RNAi) for 48 h following Ecc15 infection (G). Data represent mean ± SD (n = 3). Statistical significance was ascribed as *p<0.05; **p<0.01 and ***p<0.001 as compared to control and $p<0.05;$ $p<0.01$ and $$$p<0.001$ in comparison to respective He-Gal4. Scale bar: 20 μm. doi:10.1371/journal.pone.0088181.g009
Figure 10. Schematic representation of Cr(VI)-induced alterations on cellular immunity in D. melanogaster. Cr(VI) altered cellular innate immune response through $O_2^•−/\text{ONOOC}^−$ mediated oxidative stress in the hemocytes of exposed organism. The induction of oxidative stress leads to caspase-3 activation vis-à-vis apoptosis in the hemocytes which results into reduction in total hemocyte population in exposed organism. The altered immunity was manifested by decreased resistance of these organisms against pathogenic Ecc15 infection. Cr(VI) induced suppression of cellular immunity was subsequently modulated/rescued by over-expressing sod in Drosophila hemocytes.

Discussion

The present study explored the potential of a widely used environmental chemical, Cr(VI), to alter cellular innate immune response using a Drosophila model. Cellular immunity of Drosophila comprises hemocytes [41], therefore, the observed reduction in total hemocyte count in Cr(VI) exposed Drosophila indicates adverse impact of Cr(VI) on cellular immunity. This is corroborated with earlier studies on different model organisms [13,42]. The altered hemocyte population in exposed Drosophila was found to be consistent with the down-regulation of hemese, which is expressed in the hemocytes of Drosophila larvae [43], in Cr(VI) exposed organism. In addition, phagocytosis has been considered as one of the functional criterions which find significance in order to assess the immunological impact of environmental chemicals [36,44] on cellular immune function of exposed organism. Therefore, the observed impairment in the ability of hemocytes isolated from Cr(VI) exposed Drosophila to phagocytose GFP-labeled E. coli indicated that exposure to Cr(VI) has resulted into functional alteration in the hemocyte-mediated immune function. In this regard, previously it has been demonstrated that heavy metals affected the phagocytic activity of the immune cells in vitro [45,46].

The observed alteration in cellular immune response, therefore, may weaken the immune defense of Cr(VI) exposed organism due to reduced immune function since chemical induced impairment in innate defense mechanism was suggested to alter the resistance of exposed organism against microbial infection [3,47]. The bacterial strain, Ecc15, is a natural pathogen of the organism and is reported to induce global immune response in Drosophila by natural infection [48]. Thus, enhanced susceptibility against Ecc15 infection confirms the immuno-compromised situation of Cr(VI) exposed Drosophila larvae. Thus, reduction in total hemocyte population, decreased expression of hemese, impaired phagocytic activity and weakened resistance against bacterial infection confirms Cr(VI) induced alteration in cellular immune defense of exposed organism.

Since we observed that Cr(VI) affects cellular immune response in exposed organism, the likely reason was explored. In a cellular system, metabolic reduction of Cr(VI) to its lower oxidation states has been reported to generate ROS [9]. The latter has been causally linked to accelerated apoptotic cell death [37]. Therefore, we argued that increased ROS generation in the hemocytes of Cr(VI) exposed Drosophila larvae would increase the percent of apoptotic cell population concurrent with significantly increased level of DEVDase activity that eventually may negatively affect the health of hemocytes. The levels of ROS were measured both in terms of $O_2^•$ as well as $H_2O_2$ generation wherein higher level of DHE fluorescence as compared to DCF suggests that Cr(VI) induced generation of $O_2^•$ plays a prominent role in exerting adverse effects of the chemical on Drosophila hemocytes. Our observation was supported by a previous in vitro study wherein $O_2^•$ as the major ROS was shown to be associated with Cr(VI)-induced apoptosis [9]. Besides $O_2^•$ as one of the ROS for the observed adverse effects, it is quite likely that other reactive species may influence the above events. Since dismutation of $O_2^•$ leads to the formation of $H_2O_2$, we inhibited $H_2O_2/OH$ generation in the hemocytes of exposed organism by using an anti-oxidant NAC. However, non-significant change in the total hemocyte count and apoptosis in the hemocytes of NAC and Cr(VI) co-exposed Drosophila larvae as compared to Cr(VI)-alone exposed organism suggests that along with $O_2^•$, there is possible involvement of other reactive species instead of $H_2O_2$ in Cr(VI) induced modulation of the cellular immunity. In this context, Szabo et al. [49] stated that the reaction between superoxide ($O_2^•−$) and nitric oxide (NO) in a biological system can produce peroxynitrite (ONOO$^−$) anion. Increased ONOO$^−$ formation as evident by increased DHR fluorescence in the hemocytes of Cr(VI) exposed Drosophila could likely be the reason for free radical induced cellular injury which was also reported previously in other cell types [49,50]. Further, to confirm the involvement of ONOO$^−$ species in Cr(VI) induced alteration of cellular immune aspects, we inhibited their generation in the hemocytes of Drosophila larvae co-exposed to Cr(VI) and L-NAME (NOS inhibitor). Cr(VI) induced adverse effects on hemocytes was shown to be significantly attenuated by L-NAME as compared to Cr(VI)-alone exposed...
group. To garner support that ONOO\textsuperscript{−} generation is responsible for Cr(VI) induced alterations of cellular immunity in exposed organism, we co-exposed Drosophila larvae to SNP (NO generator) and Cr(VI). Deteriorated hemocyte health observed in SNP-Cr(VI) co-exposed organism strengthens the possibility of ONOO\textsuperscript{−} mediated injury to the hemocytes of Cr(VI) exposed organism.

Due to generation of reactive species in the hemocytes of Cr(VI) exposed Drosophila larvae, status of anti-oxidant defense system in these cells assume biological significance. In this context, significant reduction in SOD activity in the hemocytes of exposed organism suggests that SOD plays a major role as an anti-oxidant enzyme. SOD catalyses the dismutation of O\textsubscript{2}\textsuperscript{−} radical to O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2} [15] and hence decreased enzyme activity further enhances the generation of O\textsubscript{2}\textsuperscript{−} in the hemocytes of Cr(VI) exposed organism. An increase in O\textsubscript{2}\textsuperscript{−} generation due to decreased SOD activity supports the formation of ONOO\textsuperscript{−} because of the favoured reaction between O\textsubscript{2}\textsuperscript{−} and NO in the hemocytes of Cr(VI) exposed organism. Moreover, lower level of H\textsubscript{2}O\textsubscript{2} formation in the hemocytes of Cr(VI) exposed organism supports our observation of decreased activity of SOD. Interestingly, we observed an increase in DCF fluorescence in the hemocytes of exposed organism in contrast to the observed decrease in CAT activity, which is reported to breakdown H\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O and molecular oxygen [51], that is probably due to the sensitivity of DCF against both H\textsubscript{2}O\textsubscript{2} and ONOO\textsuperscript{−} [52]. Hence, decreased SOD activity along with increased O\textsubscript{2}\textsuperscript{−} generation in the hemocytes of exposed organism enhances the adversities caused by Cr(VI). In addition, decreased SOD activity in the hemocytes of exposed organism might be due to ONOO\textsuperscript{−} mediated nitration of SOD which may also reduce the enzyme activity. An increased SOD activity in the hemocytes of Cr(VI) exposed organism following inhibition of ONOO\textsuperscript{−} generation further confirmed the above (Fig. S6). Along with decreased activities of all the tested anti-oxidant enzymes and increase in lipid peroxidation in the hemocytes of Cr(VI) exposed organism, diminished level of TAC further indicates a deterioration in over-all anti-oxidant defense system vis a vis increased oxidative stress in the hemocytes of exposed organism. Thus, generation of O\textsubscript{2}\textsuperscript{−} at higher concentrations of Cr(VI) could be accounted for the oxidative damage to the hemocytes of exposed organism concomitant with increased cell death leading to the down-regulation of cellular immune response.

Based on our observations of increased generation of O\textsubscript{2}\textsuperscript{−} along with significantly decreased SOD activity in the hemocytes of exposed organism, we hypothesized that over-expression of Cu/Zn SOD in the hemocytes of Drosophila larvae would provide protection to these cells from Cr(VI) induced adversities. We, therefore, over-expressed sod in the hemocytes of Drosophila in a targeted manner using He-Gal4 driver. Contrary to the suppression of cellular immune response in Cr(VI) exposed Oregon R\textsuperscript{+} and in He-Gal4 strains, significant improvements evident in the sod over-expressed strain (He-Gal4>UAS-Sod), suggested sod governed underlying mechanism of Cr(VI) induced alteration in cellular immunity. In this context, less ROS generation, decreased oxidative stress, appearance of lesser number of apoptotic cells, decreased DEVDase activity and increased survival against Ecc\textsubscript{15} pathogenicity was observed in the sod over-expressing strain. Enhanced SOD activity in the over-expressing strain would cause dismutation of O\textsubscript{2}\textsuperscript{−} radicals, the major ROS species, shown to be involved in Cr(VI) induced adversities in the hemocytes and in the process would provide beneficial effect on cellular innate immune response of the organism. Our study finds support from an earlier study by Markovsky et al [33] which has demonstrated that sod can play an important role in adaptive immune response. The observed rescuing effect of sod over-expression against Cr(VI) induced oxidative injury to the hemocytes of exposed organism prompted us to further assess a situation when SOD is knocked down genetically from the organism. A previous study showed that Saccharomyces cerevisiae sod1 deletion strain was remarkably more sensitive to adverse effects of Cr(VI) [54]. In the same context, our observations of increased oxidative stress and apoptotic cell death in Cr(VI) exposed He-Gal4>UAS-Sod RNAi strain along with poor resistance displayed by these organisms against bacterial infection validates our hypothesis of protective role of SOD on cellular immune response of exposed Drosophila. In parallel, similar levels of metal intake in the hemocytes of exposed organism irrespective of strain difference indicate that the observed pattern is independent of strain variation. In spite of comprehensive demonstration of the protective role of SOD in improving the hemocyte health in Cr(VI) exposed organism, possibilities of other cellular machineries taking up parallel roles in this context cannot be ruled out.

Conclusions

This study provides evidences that the mechanism of Cr(VI) induced alteration of Drosophila cellular immune response involves O\textsubscript{2}\textsuperscript{−}/ONOO\textsuperscript{−} mediated oxidative stress in the hemocytes of Drosophila wherein over-expression of an anti-oxidant gene, sod, could ameliorate the Cr(VI) induced adversities by preventing oxidative injury (Fig. 10). Based on the conserved pathways for innate immunity existing in Drosophila and mammals including human, the present study enhances the broad understanding of chemical induced alterations on immunity in higher organism. Further, Drosophila is recommended as a functional in vivo model for testing the impact of environmental chemicals on innate immune response with minimum ethical concern.

Supporting Information

Figure S1 Standardization procedure of sample volume for superoxide dismutase (SOD) activity in Drosophila hemocytes. The SOD activity was standardized in Drosophila hemocytes according to the assay procedures described by McCord [27]. In this assay, SOD activity of a sample was measured as the percent inhibition of reduction in cytochrome C at 550 nm. Volume of the sample required in this assay should produce the amount of inhibition in the 40–60% range. The assay was standardized for Drosophila hemocytes by plotting the percentage inhibition against the volume of hemocyte sample (µl). The response curve suggests that 40–60% inhibition was observed by using 30 and 40 µl of hemocyte sample where 40 µl sample provides the maximum inhibition in the desired range. Therefore, this sample volume was used for further experiments to calculate SOD activity.

(TIF)

Figure S2 Optimization procedure for total anti-oxidant capacity (TAC) by ABTS decolorization assay in Drosophila hemocytes. ABTS decolorization assay was optimized in the hemocytes of Drosophila to calculate total anti-oxidant capacity with respect to the trolox standard. In this assay, the anti-oxidant capacity of a sample was measured as the percentage inhibition of the absorbance of ABTS radical cation (ABTS\textsuperscript{+}) at 734 nm. The concentration-response curve for the standard reference data was obtained by plotting the percentage inhibition of ABTS\textsuperscript{+} against trolox standard (µM) (A). The assay was then optimized for Drosophila hemocytes by plotting the percentage inhibition of ABTS\textsuperscript{+} against the volume of hemocyte sample (µl) (B). The dose-response curve suggest that 60-80% inhibition of
ABTS$^+$ was observed using 10 µl of hemocyte sample. Therefore, this sample volume was used for further experiments.

(TIF)

Figure S3 Reduced expression of hemese in Cr(VI) exposed Drosophila larvae. Relative expression of hemese in Oregon R larvae which were exposed to Cr(VI) for 24 and 48 h by qRT-PCR assay (A). Graph showing relative expression of hemese in He-Gal4, UAS-Sod, He-Gal4+/UAS-Sod, UAS-Sod RNAi and He-Gal4+/UAS-Sod RNAi larvae exposed to Cr(VI) for 48 h (B). Data represent mean values of three independent experiments (20 larvae in each). All the expression values were normalized to experimental endogenous control gapdh. Statistical significance was ascribed as \( **p<0.01; ***p<0.001 \) in comparison to control and \( \dagger\dagger p<0.01; \dagger\dagger\dagger p<0.001 \) in comparison to respective He-Gal4.

(TIF)

Figure S4 Concentration optimization of NAC, L-NAME and SNP for their exposures to D. melanogaster larvae. The concentration of N-acetylcysteine (NAC), N-nitro-L-arginine methyl ester (L-NAME) and sodium nitroprusside (SNP) was optimized in the hemocytes of Drosophila by DCF or DHR fluorescence by drawing concentration response curve for each chemical. The percent inhibition of DCF fluorescence for NAC and percent inhibition/generation in/of DHR fluorescence for L-NAME and SNP was measured in Drosophila hemocytes to calculate the concentration of the above chemicals to be used for exposures to D. melanogaster larvae. The dose-response curve of NAC (A) was plotted as normalized percent inhibition in DCF fluorescence as against 20.0 µg/ml of Cr(VI) while the same for L-NAME (B) and SNP (C) were plotted as normalized percent inhibition/generation in/of DHR fluorescence. The concentration of each chemical showing maximum/optimum inhibition in DCF/DHR or generation of DHR fluorescence was used for further experiments.

(TIF)

Figure S5 Measurement of colony forming units (CFU) in Cr(VI) exposed organisms. Ten infected larvae were surface-sterilized with 70% ethanol after rinsing them with water. Bacterial persistence was then measured by plating larval homogenate on LB medium after 1 h of infection with E. coli 15380 of each chemical showing maximum/optimum inhibition in

(TIF)

Figure S6 Determination of SOD activity in the hemocytes of Drosophila larvae co-exposed to Cr(VI) and NAC/L-NAME/SNP. Graphical representation of SOD activity in the hemocytes of 20.0 µg/ml Cr(VI) exposed Oregon R larvae along with 10 mg/ml N-acetylcysteine (NAC) or 100 mM N-nitro-L-arginine methyl ester (L-NAME) or 50 µM sodium nitroprusside (SNP) after 24 and 48 h respectively. Significant differences were determined as \( **p<0.01; ***p<0.001 \) in comparison to control and \( \dagger\dagger p<0.05; \dagger\dagger\dagger p<0.01; \dagger\dagger\dagger\dagger p<0.001 \) as compared to 20.0 µg/ml Cr(VI) exposure.

(TIF)

Table S1 Chromium level (ng/ml) in the hemocytes of different *Drosophila* larvae.

(DOC)

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Author Contributions

Conceived and designed the experiments: PP MZA DKC. Performed the experiments: PP AKS RCM DKC. Analyzed the data: PP AKS DKC. Contributed reagents/materials/analysis tools: PP AKS DKC. Wrote the paper: PP DKC.

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