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Delineating the Therapeutic Effectiveness of Allicin on Eryptosis During Chronic Pb^{2}+ Exposure in Mice

KEYWORDS: Lead (Pb^{2}+), red cell survivability, oxidative stress, allicin

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ABSTRACT *Chronic Pb^{2}+ intoxication to erythrocytes causes anaemia due to early red cell death which primarily accounts for generation of free radicals followed by waning of antioxidant system and development of oxidative stress. Garlic is a well known plant product which imparts beneficiary effect against different diseases. It is a store house of different active compounds and among which allicin has most potent antioxidant ability and metal scavenging capacity. It also protects the cell against lipoperoxidative damages. In present study, we evaluated the therapeutic efficacy of allicin against erythrocyte death after Pb^{2}+ exposure. Results suggested that allicin treatment in Pb2+ exposed mice exhibited sharp decline in PS exposure and increase in erythrocyte intracellular GSH content followed by downregulation of lipid and protein oxidations. Findings also indicated that allicin has the ability to restore red cell lifespan during chronic Pb^{2}+ exposure.*

INTRODUCTION

Lead (Pb^{2+}) is a common environmental pollutant which has ranked second in the list of hazardous substances for several years [ATSDR, 2007]. Modern industrialization and human activities are most potent source of toxic Pb^{2+} to the environment. Children and pregnant women are most vulnerable to Pb^{2+} exposure even at low concentration [Godwin *et al*., 2001, Patrick *et al*., 2006]. Erythrocytes are the primary victim of Pb^{2+} toxicity after its systemic absorption in physiological system leading to development of chronic anaemia [Flora *et al*., 2004, Gupta *et al*., 1997, Patrick 2006, Weiss *et al*., 2010]. Blood Pb^{2+} accounts for about 90% of total body Pb^{2+} burden after acute exposure and more than 95% of blood Pb^{2+} is accumulated in erythrocytes [ATSDR, 2007]. Chronic Pb^{2+} accumulation causes severe oxidative insult due to ROS generation [Mandal *et al*., 2012]. Loss of cellular thiol content occurs due to formation of Pb^{2+}-sulphahydryl protein adducts leading to dysregulation of enzymatic activities. Eryptosis or apoptosis of erythrocyte is the ultimate outcome of Pb^{2+} induced toxicity caused by PS externalization [Mandal *et al*., 2012].

Current available therapeutics against Pb^{2+} intoxication is administration of antioxidants and chelators. Antioxidant therapy is partially successful since it only provides protection against oxidative stress but cannot chelates heavy metals [Liao *et al*., 2008, Tarhan and Tuzmen 2000]. On the other hand, chelators like meso-2,3-dimercaptosuccinic acid (DMSA) and monoisoamyl DMSA (MiADMSA) often failed to remove Pb^{2+} from intracellular environment because of their hydrophilicity [Friedheim *et al*., 1978; Saxena and Flora, 2004].

Application of phytochemicals as an alternative medicinal source against several stress and inflammatory diseases has become very prominent in nowadays due to their negligible side effects. Garlic (*Allium sativum* Linn.) is well known for its medicinal impacts [Ercal *et al*., 2001]. Aged extracts of garlic or its active compound has antioxidant activity and chelating efficacy against heavy metal toxicity [Cha *et al*., 1987].The active ingredients of garlic include lipophilic sulfhur-bearing compounds which have the potentiality to restore thiol in cellular protein architecture.

In this context, we aimed to determine the therapeutic effectiveness of allicin, one of the primary active compounds of garlic, against Pb^{2+} induced intoxication. For this study, we submitted female BALB/c mice to a chronic exposure of Pb^{2+} dose and analyzed different biomarkers and enzymes.

MATERIALS AND METHODS

Chemicals

Allicin was procured from Santa Cruz Biotechnology (CA,

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USA), protease inhibitor cocktail, set III, (EDTA free) was purchased from Calbiochem (IMD Bioscience, Inc, La Jolla, CA); 3'-(phydroxyphenyl) fluorescein (HPF) was obtained from Invitrogen Molecular Probes (Eugene, OR). Annexin V (AnV)-FITC apoptosis detection kit was purchased from BioVision (Mountain View, CA). Radioactive sodium chromate (labelled with ^{51}Cr; sp act 94.2Ci/g) was procured from Board of Radiation and Isotope Technology (Mumbai, India). All other fine chemicals (unless mentioned) were purchased from Sigma Aldrich Corporation (St Louis, MO).

Animals and treatment

Animal experiments were carried out in female BALB/c mice having an average body weight of 12–15g. Animals were bred and maintained in the animal house of Vidyasagar College, Kolkata, on a 12h light/darkness cycle with *ad libitum* access to food and water. Lead acetate [$Pb(CH_3COO)_2$] was dissolved in water and administered to animals daily through oral gavage. The animals were exposed to 10mg of Pb^{2+} kg^{-1} body weight (bw) for a period of 15 days. Treated groups received allicin daily at a dose of 5, 10 and 15mg kg^{-1} bw respectively for a period of 10 days after termination of Pb^{2+} exposure.

Erythrocyte isolation and membrane preparation

Whole blood from mice was collected in heparinized tubes by puncturing the heart muscle after euthanization. Plasma was separated from erythrocytes by centrifugation at 900 x g for 10min following the standard procedure. The isolated packed cells were washed for three times with isotonic PBS (pH 7.4). For membrane preparation, packed cells were lysed overnight in hypotonic PBS (5mM), at pH 7.4. In the next day, lysed cells were centrifuged at 27,000 x g for 30min at 4°C. This process was repeated for three times until the erythrocyte membrane become free from haemoglobin [Dodge *et al.*, 1963]. The erythrocyte membrane protein was quantified by following the standard procedure of Lowry *et al.*, 1951 using bovine serum albumin as a standard index.

Analysis of cell death assay

Exposure of PS on the exofacial surface of RBC membrane was measured by flow cytometric procedure based on its binding with the FITC conjugated annexin V. Briefly, erythrocytes were diluted 1:100 (2 x 10^8 erythrocyte/ml) to a final volume of 0.25ml in a HEPES-buffered saline (HBS) consisting of 10mmol/L HEPES–Na pH 7.4, 136mmol/l NaCl, 2.7mmol/l KCl, 2mmol/l $MgCl_2$, 1mmol/l NaH_2PO_4, 5mmol/l glucose, 5mg/ml bovine serum albumin and 2.5mmol/l $CaCl_2$. Next, 60ng/mL FITC–annexin V was added and the sample was incubated for 30min at room temperature in the dark. After incubation, cells were centrifuged at 1000 x g for 5 min. Pelleted RBCs were again resuspended in HBS and subjected to flow cytometer (BD Bioscience, Mountain View, CA). Ten thousand events per sample were acquired for the data analysis [Fabisiak *et al.*, 1999].

Estimation of intracellular ROS content

Accumulation of hydroxyl radical (OH^-) was detected by incubating 5% red cell suspension with HPF for 15min at 37°C. Erythrocytes were then isolated and washed twice with HEPES buffer (pH 7.4). The fluorescence of the samples was estimated in a microplate fluorimeter (Perkin Elmer, Model LS55, Llantrisant, UK) at excitation: 485nm and emission: 520nm [Hempel *et al.*, 1999].

Haematocrit estimation

Percent hematocrit or packed cell volume was measured by collecting blood from tail vein. Blood was then collected in heparinized capillary tube and centrifugation was subsequently done at 10,000 x g for 10min. Finally, proportion of packed erythrocytes and total blood volume was calculated.

Determination of osmotic fragility of red cell

The osmotic fragility of erythrocytes was determined by measuring the haemolysis spectrophotometrically at 640nm in hypotonic saline. The extent of haemolysis of the cells in saline was assessed in comparison to the lysis of the same volume of red cells in distilled water [Suboh *et al.*, 2004].

Analysis of erythrocyte survivability

Red cell survivability was measured from the half-life of erythrocyte over time [Southerland *et al.*, 1955]. Briefly, [^{51}Cr]-labelled sodium chromate was injected into mice at the dose of 20µCi/kg. Blood was collected from mice and was washed in 20mM HEPES–Tris (pH 7.4) and then lysed by adding distilled water. The radioactivity of lysates was measured using a gamma-counter (K2700B/ECIL). Count obtained on the first day of injection was considered as 100% value. The radioactivity present in each sample on any subsequent day is calculated with respect to this initial 100% value as a percent of initial activity. The day at which 50% radioactivity disappeared was termed as $t_{1/2}$.

Assay of intracellular GSH level

Determination of intraerythrocytic GSH was initiated by cell lysis with 0.1% EDTA solution. Next precipitating reagent (containing 0.21M metaphosphoric acid, 5.4mM EDTA and 5.13M NaCl) was added to it and the final solution was allowed to stand for 5min before filtration. Finally 0.3M disodium hydrogen phosphate and 0.04% DTNB (0.04%) in sodium citrate solution (10g/l) were consecutively added to the filtrate. Change in colour was analyzed at 412nm [Tietze 1969].

Determination of cellular TBARS and protein carbonyl content

TBA reactive substances (TBARS) was measured by adding 1ml of 10mM Tris–HCl buffer (pH 7.4) to 0.4mg of membrane protein (in 0.9% saline, pH 7.4) followed by addition of 2ml TBA–TCA reagent (15% TCA and 0.4% TBA) and the reaction mixture was boiled for 15min. After centrifugation the absorbance of supernatant was measured at 532nm. Quantification was based upon the molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ and was expressed in nmoles/mg protein [Buege and Aust 1978]. Protein carbonyl was measured as a marker of protein oxidation using 2,4- dinitrophenylhydrazine [Keenoy *et al.*, 1999]. The carbonyl content was calculated from absorbance at 365nm, using a molar extinction coefficient of 22 × 10^2 $M^{-1}cm^{-1}$.

Statistical analysis

All experimental data were given as mean ± S.D. Differences between two groups were determined by unpaired Student's t-test. For multi group comparisons, analysis of variance was computated by the ANOVA followed by Student–Newman–Keuls test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of allicin on reduced lifespan of erythrocyte during chronic Pb^{2+} intoxication

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[Table 1 about here]
Chronic Pb^{2+} intoxication is highly associated with red cell death causing anemia. To infer the therapeutic effect of allicin on red cell survivability we treated Pb^{2+} affected mice with different concentration of allicin (5, 10 and 15mg/kg bw) and measured parameters indicative of red cell death and development of anemia. Table 1 suggested there was a significant reduction of haematocrit level in Pb^{2+} exposed mice which was very significantly restored ($p<0.001$) towards control level after allicin treatment at a dose of 15mg/kg bw. The reduction in haematocrit level in Pb^{2+} exposed mice was associated with marked decay in erythrocyte lifespan (Table 1). Allicin (20mg/kg bw) treatment was most successful in prolonging the lifespan of red cell thereby preventing early hemolysis in Pb^{2+} intoxicated mice. Alternation in membrane permeability and its destabilization induced ionic imbalance leads to osmotic damage is also one of the criteria leading to red cell death. Our result (Table 1) suggested that there was a severe osmotic lysis of Pb^{2+} affected red cells. Treatment with allicin (20mg/kg bw) was also able to reduce significantly the osmolysis after Pb^{2+} exposure.

Therapeutic effectiveness of allicin on eryptosis after Pb^{2+} exposure
[Figure 1 about here]
Reduction in haematocrit level and development of anemia in Pb^{2+} intoxicated mice are the consequences of erythrocyte death due to PS externalization. Figure 1 demonstrated that there was 21.5% of Annexin V bound positive cells in Pb^{2+} exposed mice which was significantly reduced (13.3%) after allicin treatment (15mg/kg bw) clearly indicating that allicin administration played a vital role in reducing eryptosis.

Allicin treatment successfully combated ROS level and restored cellular GSH level in Pb^{2+} exposed mice

[Figure 2 about here]
Since it has been reported that ROS is one of primary contributor of red cell death during chronic Pb^{2+} intoxication, hence we became determined to investigate the status of free radicals during Pb^{2+} toxicity. Figure 2 depicted that there was significant rise in HPF fluorescence level in Pb^{2+} exposed groups indicating accumulation of OH^{-} radicals. Administration of allicin (15mg/kg bw) was able to reduce ROS levels similar towards control counterparts. Enhanced accumulation of ROS leads to waning of antioxidants and development of oxidative stress. Our result indicated that there was a concomitant rise in TBARS level in Pb^{2+} exposed group which was successfully ameliorated after allicin treatment. Again the ability of allicin therapy to inhibit protein oxidation was evident from a close correlation with lipid peroxidation. Reduced glutathione (GSH) is one of the predominant non-enzymatic antioxidant present within erythrocytes and during oxidative stress it gets oxidized to GSSG by scavenging free radicals. Our data suggested there was a marked decrease in GSH content within red cell after Pb^{2+} exposure. Treatment with allicin at a dose of 20mg/kg bw successfully restored the GSH level like that of control ones.

DISCUSSION
Anemia is probably one of the most predominant toxic effects of chronic Pb^{2+} exposure. Erythrocyte is one of the primary cell types that encounter Pb^{2+} as it enters the circulation [ATSDR 2007; Gupta and Shukla 1997]. Center for Disease Control (CDC) defines Pb^{2+} poisoning when the blood Pb^{2+} level (BLL) exceeds 10µg/dl (0.5l M) [CDC, 1997]. Chronic Pb^{2+} exposure causes severe oxidative insult in erythrocytes [Weiss *et al.*, 2010]. ROS detoxification is one of the prerequisite of red cell survival and hence a potential antioxidant system is present within erythrocyte. In our present study, erythrocytes isolated from Pb^{2+} exposed mice were associated with increased oxidative stress, as evident from gradual decline in intracellular GSH content and increase in erythrocyte TBARS levels. Radical-mediated protein oxidation may contribute to progressive membrane damage and destabilization, which eventually alter membrane permeability, resulting in osmotic fragility leading to cellular lysis [Levander *et al.*, 1977]. During severe oxidative stress PS present in the inner leaflet membrane gets externalized [McEvoy *et al.*, 1986] which was well corroborated with our present findings ultimately leading cell death and reduction in the lifespan [McEvoy *et al.*, 1986]. Data analysis of our study also suggested that eryptosis (PS externalization) is one of the major mechanism that contributes to the development of anemia during chronic Pb^{2+} toxicity [Sarkar *et al.*, 2014].

Present therapeutic approaches mostly involve the usage of heavy metal chelators in the treatment of Pb^{2+} toxicity. However, chelators like DMSA and MiADMSA cannot impregnate the cell membrane barrier and cellular micro-environment [Saxena and Flora 2004; Liao *et al.*, 2008; Kalia and Flora 2005]. While the use of antioxidants as therapeutics has been partially successful since they provides protection against free radicals only but generally cannot scavenge heavy metals.

Our aim was to explore alternative therapeutic strategy using natural product like garlic containing several essential active compounds beneficial against several diseases. Amongst many active compounds allicin is one of the most potent compounds which have been reported to fight against different pathological conditions [Bayan *et al.*, 2014]. Allicin is also known to act as a metal chelator and can protect the cell from oxidative stress because of its antioxidant property [Bayan *et al.*, 2014]. Thus we evaluated the role of allicin in the amelioration of the early death of erythrocytes during chronic exposure to Pb^{2+}.

Our data suggested efficient contribution of allicin in restoring non enzymatic antioxidants (GSH) within erythrocytes isolated from Pb^{2+} exposed mice. This might be attributed to the presence of antioxidant potential in allicin. Allicin has the efficiency to scavenge ROS as well as in the chelation of heavy metals where we also found a downregulation in lipid peroxides and protein oxides within red cells even after Pb^{2+} exposure to mice.

PS exclusively remains on the cytoplasmic face of the plasma membrane [Tyurina et.al. 2007]. Asymmetric distribution of the PS is being regulated by the ATP dependent enzyme APLT [Tyurina et.al. 2007]. During eryptosis the function of this enzyme is downregulated causing transport of PS from inner to outer leaflet [Tyurina et.al. 2007]. Our findings suggested that allicin was successful in downregulating ROS mediated PS exposure of erythrocyte which could attributes to its role in preventing anemia during Pb^{2+} exposure. Reports suggest that sulphahydryl group is essential for the maintenance of cytosolic and membrane bound enzyme activities like APLT [Ou *et al.*, 2003]. Thus allicin, a well known thiol containing compound, possibly restores the APLT activity by sensing the thiol group keading to reduction in PS externalization. Finally, revival from oxidative stress mediated osmotic damage to membrane followed by a successful recovery from cell death might be

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the probable reason for enhancement in red cell lifespan as well as restoration of Hb level as evident from increased in hematocrit level after allcin treatment to Pb^{2+} exposed mice.

CONCLUSION

Our study has demonstrated that allicin (a potent active compound of garlic) treatment at a dose of 15mg/kg bw efficiently downregulated Pb^{2+} mediated eryptosis in mice and restored the red cell lifespan along with reduction in anemia.

FIGURE LEGENDS

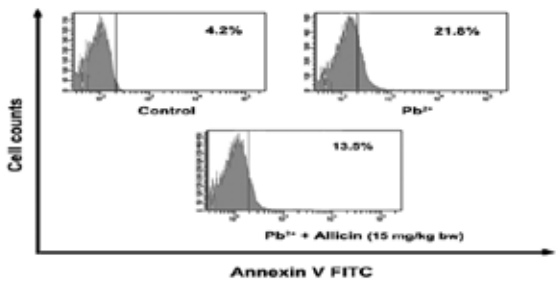

Fig: 1 Allicin treatment attenuated Pb^{2+} induced red cell apoptosis in mice. Mice were exposed to Pb^{2+} at a dose of 10mg kg^{-1} body weight for a period of 20 days. Allicin was administred at a dose of (5, 10, 15mg/kg bw) for 15 days after termination of Pb^{2+} exposure. Eryptosis via PS externalization was evaluated from histograms of Annexin V binding.

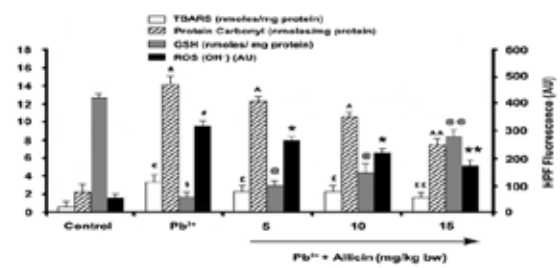

Fig: 2 Allicin administration successfully mitigated intracellular ROS and restored GSH level in Pb^{2+} exposed mice. Mice were exposed to Pb^{2+} at a dose of 10mg of kg^{-1} body weight for a period of 20 days. Allicin was administred at a dose of (5, 10, 15mg/kg bw) for 15 days after termination of Pb^{2+} exposure. Intracellular ROS, TBARS, protein carbonyl and GSH levels were estimated. For ROS assay freshly collected red cells were tagged with fluorescenct dye HPF and were analyzed spectroflurimetrically. Details of all the experimental processes have been described in the materials and methods section. Results are mean ± SD of four independent experiments with 4 animals in each group. $^{\epsilon}p < 0.01$ vs. respective age matched controls, $^{£}p < 0.05$ and $^{ff}p < 0.01$ with respect to Pb^{2+} exposed groups. $^{\&}p < 0.01$ vs. respective age matched controls, $^{\wedge}p < 0.05$ and $^{\wedge\wedge}p < 0.01$ with respect to Pb^{2+} exposed groups. $^{\$}p < 0.01$ vs. respective age matched controls, $^{@}p < 0.05$ and $^{@@}p < 0.01$ with respect to Pb^{2+} exposed groups. $^{\#}p < 0.01$ vs. respective age matched controls, $^{*}p < 0.05$ and $^{**}p < 0.01$ with respect to Pb^{2+} exposed groups.

TABLE 1

Studies of the therapeutic efficacy of different dose of allicin on haemato-pathological and red cell lifespan

	Control	Pb^{2+}	Pb^{2+} + Allicin (5mg/kgbw)	Pb^{2+} + Allicin (10mg/kgbw)	Pb^{2+} + Allicin (15mg/kgbw)
% Haema-tocrit	57.87±3.66	16.42±2.03#	21.61±0.35*	29.68±1.96*	36.84±1.05**
Red cell surviv-ability ($t_{1/2}$ In Days)	22.38±1.42	6.44±0.26#	9.98±0.15*	12.66±0.66*	16.94±0.73**
Osmotic fragility (% lysis)	38.64±2.05	79.44±3.98#	69.94±2.31*	61.26±3.55*	52.73±3.22**

All data are expressed as mean ± SD. Data are representative of 4 independent experiments with 4 animals in each group. Unpaired t-test was applied to determine the statistical significances.

Significantly different from control at $p<0.01$.

* Significantly different from Pb^{2+} Exposed at $p<0.05$.

** Significantly different from Pb^{2+} Exposed at $p<0.01$.

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