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Original Article

# α-Lipoic Acid Mitigates Arsenic-Induced Hematological Abnormalities in Adult Male Rats

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## What's Known

- Arsenic affects multiple organs by oxidative stress.
- α-lipoic acid is known to reduce such effects upon other heavy metal (lead) exposure and diabetic neuropathy amongst others. In such work, its benefits as an antioxidant were shown. Its efficacy upon arsenic exposure remains unexplored.

## What's New

- α-lipoic acid has been shown to protect arsenic mediated haematological alterations such as echinocytic transformations of erythrocytes. Total antioxidant and total oxidant status were also improved in the blood of adult male rats.
- Anti-inflammatory potential of ALA amidst arsenic insult was also observed.

## Abstract

**Background:** Arsenic toxicity is a major global health problem and exposure via contaminated drinking water has been associated with hematological and other systemic disorders. The present investigation has been conducted in adult male rats to evaluate the protective ability of α-lipoic acid (ALA) against such hematological disorders.

**Methods:** Twenty-four adult male Wister rats (b.wt.130±10g) were grouped and accordingly group I (control) received the normal diet, group II (treated) was given arsenic orally for 28 consecutive days as arsenic trioxide (3 mg/kgbw/rat/day) whereas group III (supplemented) received the same dose of arsenic along with ALA (25 mg/kgbw/rat/day) as oral supplement. Hematological profile, plasma oxidant/antioxidant status, and erythrocyte morphology were assessed. Statistical analysis was done by one-way ANOVA using SPSS software (version 16.0).

**Results:** Arsenic exposure caused reduction of erythrocyte ($P=0.021$), leucocyte ($P<0.001$), and hemoglobin ($P=0.031$) associated with echinocytic transformation as evidenced by light and scanning electron microscopic studies. The other significantly altered parameters include increased mean corpuscular volume ($P=0.041$) and lymphocytopenia ($P<0.001$) with insignificant neutropenia and eosinophilia. Altered serum oxidative balance as evidenced by decreased TAS ($P<0.001$) and increased TOS ($P<0.001$) with OSI ($P<0.001$) was also noted. The dietary supplementation of ALA has a beneficial effect against the observed ($P<0.05$) arsenic toxicities. It brings about the protection by restoring the hematological redox and inflammatory status near normal in treated rats. Arsenic-induced morphological alteration of erythrocytes was also partially attenuated by ALA supplementation.

**Conclusion:** It is concluded that arsenicosis is associated with hematological alterations and ALA co-supplementation can partially alleviate these changes in an experimental male rat model.

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**Keywords** ● Arsenic ● α-lipoic acid ● Hematology ● Erythrocytes ● Oxidative stress

## Introduction

Heavy metal toxicity due to occupational and domestic exposures raises apprehension over their potential effects on human health and the environment. Chronic arsenic toxicity, also known

as arsenicosis,[1,2] is a global health problem affecting millions of people and mainly caused by drinking of arsenic-contaminated ground water. The worst affected countries include India, Bangladesh, Pakistan, Japan, and the United States.[3]

Arsenic is a naturally occurring metalloid having four primary oxidative states: +5, +3, 0, and -3, among which the trivalent arsenite form ($As_2O_3$; As III) and the pentavalent arsenate form ($As_2O_5$; As V) are of utmost biological relevance. The inorganic trivalent species of arsenic are of great concern to the toxicologists compared to the organic one.[4] Arsenic exposure shows various systemic manifestations, such as pigmentation and keratosis of skin, bronchitis, obstructive and/or restrictive pulmonary diseases, liver disorders allied with non-cirrhotic portal fibrosis, gastrointestinal disorder, polyneuropathy, cancer of various organs like skin, lung, liver, kidney and urinary bladder, anemia etc.[1] Arsenic toxicity-acute, subchronic or chronic is known to interfere with hematopoietic and immune systems.[4] The first tissue that encounters arsenic in the body after its systemic absorption is blood.[5] Arsenic exposure may lead to its accumulation in erythrocytes and cause anemia associated with leucopenia with characteristic neutrophil depletion and thrombocytopenia.[6,7] Arsenic exerts its toxicity by inactivating numerous enzymes, including the enzymes involved in cellular energy pathways, DNA replication, and repair.[8] Apart from this, arsenic attacks cellular redox systems resulting in the generation of excess reactive oxygen species (ROS) in the form of superoxide anion ($O_2$-) and hydroxyl radical (OH) leading to substantial oxidative damages of proteins, lipids, and DNA.[4] As erythrocytes lack any replenishing machinery and have a considerably higher average life of about 120 days, it therefore can carry the impression of long-term toxic and/or pathological insult. Moreover, in vitro sodium arsenite even at a very low concentration is reported to induce apoptosis in rat bone marrow mesenchymal stem cells.[9] However, a detailed haematological picture in chronic arsenic intoxication has hardly been reported in toxicological studies.

Alfa lipoic acid (ALA), is an endogenously produced coenzyme and is being used by researchers as a dietary supplement with good bioavailability, both in humans and experimental animals.[10,11] The role of ALA as anti-oxidant and in the prevention of hematological abnormalities caused due to heavy metal toxicity (such as lead, cadmium and copper) has been reported earlier and it has been found that the co-administration of ALA with heavy metals maintains normal antioxidant activity and normalize hematological parameters, especially anemia, in rats.[12,13] ALA is also reported to prevent arsenic toxicity via protecting against oxidative damages in acute and chronic animal model[14,15] as well as in vitro studies.[16] A few studies have been undertaken to find the corrective approach of ALA on hematological alterations by heavy metals, but protective nature of ALA on blood or its components due to subchronic exposure of arsenic in rats are currently lacking. Therefore, the aim of the present study is to find out the protective role(s) of ALA, if any, on arsenic-induced hematological alterations and oxidative stress using rats as experimental animal.

## Materials and Methods

### *Animal Selection and Drug Treatment*

Subchronic arsenicosis was developed in Wister rats according to standardized protocol of our laboratory.[17] In short, a dose of $As_2O_3$ (3 mg/kg body weight/rat/day) was selected and administered per orally over a period of 28 days. ALA was dissolved in olive oil and was administered per orally once daily (25 mg/kg body weight/rat) after about 2 hours of the meal. The dose of ALA is calculated keeping the dose for human (per kg body weight) essentially as described previously.[18,19] Twenty-four adult male Wister rats weighing 130±10 g were selected for this experiment. The animals were maintained under standard laboratory conditions (14 h light: 10 h dark, 25±2°C) with free access to food and water. All animal experiments were performed according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (number: 796/ac/03/CPCSEA/18-01 dated 18.01.2013). Rats were randomly grouped into three, consisting eight rats in each group as control (group I), arsenic-treated (group II), and arsenic-treated and supplemented with ALA (group III). All the animals of groups I, II and III were provided with a normal diet composed of 71% carbohydrate, 18% protein, 7% fat, and 4% salt mixture and vitamins.[17] Both the group II and III rats were gavaged arsenic trioxide solution where only the rats of group III were given ALA per orally after the meal. To overcome the impact of any altered food intake, group I rats were pair-fed with other experimental groups II and III. Food and water intake and body weight of the rats were monitored throughout the experimental period. Rats were sacrificed in the morning of the day after last treatment.

### *Sample Collection*

Blood was collected under sterile condition from anesthetized rats by cardiac puncture and

kept in both ethylenediaminetetraacetic acid (EDTA) and heparinized vials for hematological and biochemical analyses, respectively. Some portion of EDTA-treated blood was also used for scanning electron microscopy (SEM) of erythrocytes.

*Hematological Profiling*

Complete blood counts (total and differential) and estimation of hematological indices, including total haemoglobin (Hb), packed cell volume (PCV), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) were performed using an automated cell counter (Beckman Counter, France). Neutrophil to lymphocyte ratio (NLR) and platelet to lymphocyte ratio (PLR) were calculated with the help of absolute count of neutrophil, lymphocyte, and platelet.

*Determination of Plasma Total Antioxidant Status (TAS) and Total Oxidant Status (TOS)*

Plasma concentration of TAS was estimated based on the inhibition of radical cation $ABTS^+$ [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation], which has characteristic long wavelength absorbance maxima at 734 nm and calculated from Trolox standard curve as described earlier.[20] The absorbance of the stock solution containing 7mM $ABTS^+$ (produced from 12-16 hour incubation of 7 mM ABTS and 2.45 mM potassium persulphate in 0.01 M phosphate buffer saline, pH-7.4) was adjusted to about 0.700 at 734 nm with 0.01 M phosphate buffer saline, pH-7.4. 10 µl of sample was then mixed with 1 ml diluted reagent. Change in absorbance (ΔA) was calculated from absorbance reading just before adding sample and after 6 minutes of sample addition was recorded and converted to mM Trolox equivalent. TOS was estimated according to the method of Erel,[21] which is based on the generation of colored complex of ferric ion in the presence of oxidative components and xylenol orange in acidic medium and calculated from $H_2O_2$ standard curve. In short, 140 µl of sample was added to 900 µl of reagent 1 (containing 150 µM xylenol orange, 140 mM NaCl and 1.35 M glycerol in 25 mM $H_2SO_4$ solution, pH 1.75) and mixed. The initial reading ($\Delta A_1$) was obtained from subtracting absorbance at 800 nm (secondary wavelength) from that of 560 nm (primary wavelength). 44 µl of reagent 2 (containing 5 mM ferrous ion and 10 mM o-dianisidine in 25 mM $H_2SO_4$ solution) was then added to the above mixture and incubated for 4 minutes. The final reading ($\Delta A_2$) was recorded similarly. The value of TOS was then obtained using $\Delta A$ ($=\Delta A_2-\Delta A_1$) from the $H_2O_2$ standard curve. Oxidative stress index (OSI) was calculated from the ratio of TOS and TAS {OSI=[(TOS, µM H2O2 equivalent)×100/(TAS, µM Trolox equivalent)]} according to the standard method[22] and expressed as arbitrary units. For this purpose, the result unit of TAS was changed to µM Trolox equivalent.

*Light Microscopy*

Peripheral blood smear was prepared on grease free glass slides and photographed using Zeiss light microscope (Zeiss, Thornwood, NY) and progress capture Pro 2.5 software (GENOTYPIC Optical Systems, GmBH, Jena, Germany) using 1000× magnification after staining with the Leishmann stain.

*Morphological Studies on Erythrocytes Using SEM*

Erythrocytes were processed for morphological studies by SEM, essentially as described previously.[23] Briefly, erythrocytes were directly fixed overnight with 2.5% glutaraldehyde solution in PBS, pH 7.2, and post-fixed by keeping overnight in 1% osmium tetraoxide in the same buffer. The suspensions were dehydrated in an ethanol series. After drying with carbon dioxide by the critical point method and sputter coating with gold, samples were examined on a SEM (VEGAII LSU, TESCAN, Czech Republic).

*Statistical Analysis*

Data were represented as mean±standard error of mean. One-way ANOVA followed by Tukey's HSD post hoc analysis was performed to check any statistical difference between the parameters of the studied groups. Two tail $P<0.05$ was considered as significant. Statistical analysis was carried out using the statistical program packages SPSS version 16.0 for Windows (SPSS Inc., Chicago, USA).

## Results

*Hematological Profiling*

Assessment of hematological profile indicated that hemolytic and inflammatory responses are the primary events associated with arsenic toxicity in rats after arsenic treatment (table 1). ALA supplementation revealed protection against arsenic-induced hematological changes. A significant reduction of erythrocyte count ($P=0.021$) associated with decreased total Hb content ($P=0.031$) was noticed in arsenic-treated rats compared to that of control. MCV was found to be significantly increased ($P=0.041$)

**Table 1:** Effect of ALA supplementation on hematological profile of $As_2O_3$ treated male rats

| Parameters | Control | Arsenic | Supplemented | One way ANOVA (P) |
|---|---|---|---|---|
| Erythrocyte ($10^6$/µl) | 8.06±0.144 | 7.31±0.182* | 7.59±0.193 | 0.025 |
| Leucocyte ($10^3$/µl) | 9.83±0.507 | 5.67±0.506* | 7.23±0.454# | <0.001 |
| Platelet ($10^3$/µl) | 651.00±48.137 | 498.83±33.297* | 627.00±20.930 | 0.019 |
| Hemoglobin (gm/dl) | 13.53±0.240 | 12.60±0.171* | 13.12±0.157 | 0.036 |
| Mean corpuscular volume (fl) | 51.66±0.683 | 55.20±1.367* | 54.38±0.473 | 0.040 |
| Mean corpuscular hemoglobin (pg) | 16.81±0.349 | 17.82±0.372 | 17.43±0.229 | 0.120 |
| Mean corpuscular haemoglobin Concentration (gm/dl) | 32.54±0.483 | 32.32±0.601 | 32.07±0.542 | 0.830 |
| Neutrophil count ($10^3$/µl) | 1.94±0.324 | 1.23±0.193 | 1.00±0.035# | 0.022 |
| Lymphocyte count ($10^3$/µl) | 7.12±0.453 | 3.52±0.284* | 5.30±0.263#,$ | <0.001 |
| Monocyte count ($10^3$/µl) | 0.54±0.159 | 0.52±0.125 | 0.52±0.166 | 0.993 |
| Eosinophil count ($10^3$/µl) | 0.11±0.026 | 0.16±0.013 | 0.11±0.009 | 0.067 |
| Basophil count ($10^3$/µl) | 0.11±0.060 | 0.06±0.017 | 0.06±0.010 | 0.572 |
| Neutrophil to lymphocyte ratio | 0.29±0.062 | 0.39±0.056 | 0.19±0.012$ | 0.037 |
| Platelet to lymphocyte ratio | 91.77±5.041 | 146.52±15.728* | 119.78±6.994 | 0.007 |

Data represented as mean±standard error of mean. Tukey HSD post hoc analysis, P<0.05; *Control versus treated; #Control versus supplemented; $Treated versus supplemented

in arsenic-treated rats compared to that of control, suggesting macrocytosis. Hematocrit (P=0.999) and other red blood cell indices viz. MCH (P=0.105) and MCHC (P=0.954) showed insignificant variations. In addition, the toxic effect of arsenic also caused significant neutropenia (P=0.022), lymphocytopenia (P<0.001), and thrombocytopenia (P=0.023) indicating compromised hemostatic and immune system. Eosinophil count shows insignificant increase (P=0.123) whereas monocyte (P=0.993) and basophils (P=0.684) shows insignificant variations after arsenic treatment. Neutrophil to lymphocyte ratio (NLR) and platelet to lymphocyte ratio (PLR), the derived hematological indices representing inflammation, were also altered after arsenic treatment. Though NLR showed insignificant elevation (P=0.330), PLR was found to be significantly increased (P=0.005) in arsenic-treated rats. ALA treatment has yielded partial protection from hematological insults. ALA supplementation resulted in partial restoration of erythrocyte (P=0.503), Hb (P=0.180), and MCV (P=0.807), though insignificant, but leads to significant restoration of lymphocyte count (P=0.006) and thereby partial restoration of total leucocyte count (P=0.093) compared to the arsenic-treated group, but failed to achieve normal status. Neutrophil (P=0.748), eosinophil (P=0.086), basophil (P=0.986) and monocyte (P=0.999) count does not show any significant improvement. It also significantly reduces the NLR value (P=0.029) associated with a non-significant reduction of PLR (P=0.195) compared to the arsenic-treated group.

*ALA Prevents Arsenic-Induced Morphological Alteration of Erythrocytes*

Hematological alterations were found to be accompanied by poikilocytosis, i.e. morphological alteration of erythrocytes, characterized by the formation of echinocytes and spherocytes and such changes were not seen in the control group (figure 1A and B). The peripheral smear of arsenic-treated rats contains about 19.77% echinocytes and 6.78% spherocytes compared to 1.98% echinocytes and 0.99% spherocytes in control. This morphological response was found to be partially prevented by ALA supplementation (figure 1C). ALA co-administration reduces the echinocytic content to about 4.69% and that of spherocytes to 1.56%.

Arsenic-induced echinocytic transformation of erythrocyte was confirmed by SEM analysis, which showed distinct membrane blebbing, increased number of spherocytes and microcytosis, but these were almost absent in the control group (figure 2A and B). Interestingly, ALA supplementation gave substantial protection from arsenic-induced echinocytic transformation (figure 2C).

*Arsenic Affects Plasma Redox Status*

Arsenic treatment caused a significant oxidative stress in rats as evident from the elevated plasma TOS (P<0.001), OSI (P<0.001) and reduced plasma antioxidant status (P<0.001) compared to the control group. ALA, known for its antioxidant potential, was seen to significantly prevent oxidative stress by lowering plasma TOS (P<0.001) and OSI (P<0.001) status in spite of arsenic supplementation. ALA also helped in

restoring the antioxidant capacity as shown by a significant increase in plasma TAS (P=0.005) in LA supplemented group compared to the arsenic group (figure 3).

## Discussion

In the present study, hematological alterations and associated redox imbalance were seen due to arsenic treatment, which were found to be maintained near normal status after ALA supplementation; also in favour of the contemporary report on the protective potential of ALA against arsenic toxicity.[24] The counts of erythrocyte, leucocyte, and platelet along with total hemoglobin content, which were depleted due to arsenic insult showed to be prevented on ALA supplementation. Although no significant prevention was noted in other hematological indices, but an order of control was seen in this regard due to ALA supplementation in arsenic-treated rats. This finding is in partial agreement with related recent reports of hematoprotective effect of ALA against heavy metal toxicity,[12,13] but again, it is to be noted that in these studies ALA is used in combination with other drugs. The partial restoration of hematological parameters after ALA supplementation is in agreement with previous reports, which show that despite its antioxidant potential, ALA fails to significantly improve hematological parameter.[25] Arsenic-neutrophil interaction[26] and its deleterious effect on the neutrophils, which potentiates its anticancer activity is well documented in the literature.[8,27] Neutropenia is so profound in arsenic-treated rats that even the ALA supplementation fails to restore its count even to near normal. NLR and PLR are two derived measures obtained from the neutrophil, lymphocyte, and platelet absolute counts are recently reported as the marker of systemic inflammation and related with disease

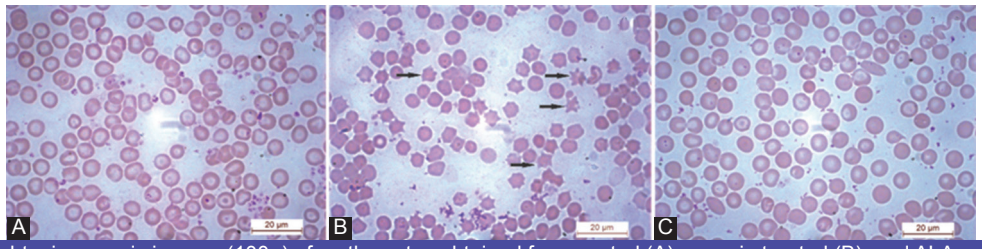


**Figure 1:** Light microscopic images (100×) of erythrocytes obtained from control (A), arsenic-treated (B), and ALA-supplemented (C) rats. Echinocytes were shown with a black arrow.

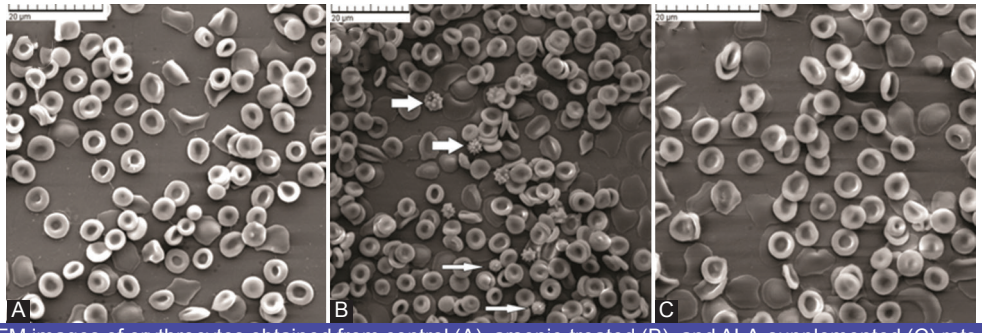


**Figure 2:** SEM images of erythrocytes obtained from control (A), arsenic-treated (B), and ALA-supplemented (C) rats. Echinocytic and microcytic spherocyte transformations of erythrocytes are shown by thick and thin white arrow, respectively.

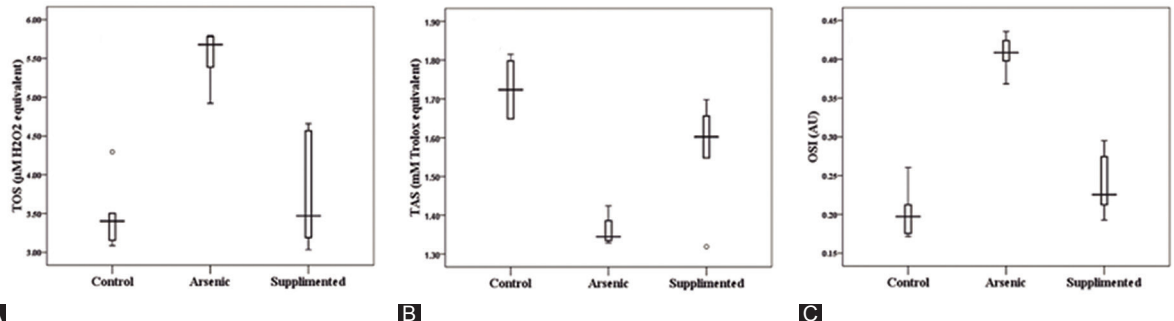


**Figure 3:** Plasma redox status of control (A), arsenic-treated (B), and ALA-supplemented (C) rats.

outcome.[28] Recent reports also suggest that NLR is elevated in chronic stress in rats and it can serve the purpose of a physiological marker in experimental animals.[29]

Both NLR and PLR were found to be elevated after arsenic exposure indicating systemic inflammation caused by arsenic toxicity. ALA administration significantly reduces these two scores in spite of arsenic exposure even below the status in the sham control group. The differential responses of NLR and PLR in the case of arsenic treatment and ALA supplementation suggests that in the case of prognosis of arsenic toxicity, a combination of these two should be taken into account as also suggested by other authors.[30] The anti-inflammatory activity of ALA in this model of arsenicosis also supports the anti-inflammatory potential as reported[31] in both acute and chronic model in rats.

Arsenic imposes its damaging effects via impairment of enzyme by forming strong complexes with thiols. It also generates ROS during its metabolism in cells leading to redox imbalance and enhanced lipid peroxidation.[32] Arsenic-induced oxidative stress and toxicity is bring about by generated free radicals and potentiated by arsenic metabolites especially the organic derivatives of arsenic. Dimethylarsine (a trivalent form of arsenic) can react with molecular oxygen to form a (CH3)2As. radical and superoxide anions. This (CH3)2As. is converted to (CH3)2AsO. radical by adding another molecule of oxygen. All these free radicals along with others, like $OH^{\cdot}$ ultimately damage various cellular macromolecules, including proteins and DNA.[33] Generation of ROS, beyond the body's endogenous antioxidant balance was known to cause redox imbalance favoring an oxidative milieu.[34] Noticeable increase in plasma TOS associated with a decrease in plasma TAS in comparison to that of the control group indicates a state of oxidative stress due to arsenic toxicity, which further supports the notion that redox imbalance is one of the significant events leading to damages due to arsenicosis. These alterations lead to marked elevation of OSI, suggesting a fair reduction of the antioxidant system parallel to increased ROS production after arsenic exposure. ALA, a naturally occurring antioxidant and coenzyme of many enzymes, includes key enzymes of energy metabolism pathway.[10,11] ALA and its derivative dihydrolipoic acid (DHLA) quench a number of reactive oxygen species in both lipid and aqueous phases, chelate transition metals, prevent membrane lipid peroxidation along with protein damage via interactions with vitamin C and glutathione and thus they are considered as potent antioxidants.[35] ALA shows a significant prevention of redox alteration induced by arsenic treatment, as confirmed by the TOS, TAS, and OSI status in the supplemented group and this is in accordance with other previous reports in this field.[36] These findings/observations suggest that the beneficial effect of ALA supplementation in preventing the hematological alteration and inflammatory changes in a rat model of arsenicosis may be attributed to its antioxidant potentials as also suggested by others.[31]

Microscopic study has shown that arsenic exposure leads to poikilocytic response and reduced discocyte content in rats. The observed major shape transformations being echinocytic and spherocytic, which are in agreement with the previous reports on arsenic affected human populations.[37,38] SEM image analyses confirm these findings and also indicate microcytosis in the arsenic-treated groups. Membrane blebbing has also been observed in the pheripheral erythrocyte due to arsenic toxicity. These changes in erythrocyte morphology may be attributed to increased oxidative stress and inflammatory status due to arsenic exposure, which is suggestive of eryptotic changes.[39] Oxidative stress within erythrocytes that occurs due to arsenic exposure and accumulation is most likely responsible for membrane injury, methemoglobin formation, osmotic fragility, and destruction of cells.[6] ALA treatment ameliorates poikilocytic effect of arsenic and increases discocyte content, reduces microcytosis and membrane blebbing in the peripheral blood. This may be explained by the unique ability of the ALA which could neutralize free radicals within aqueous and lipid regions of the cell.[40] This uniqueness of the ALA, allows it to be transported easily across the cellular membrane and thereafter alleviate the ROS induced damages by its antioxidant capacity and/or by the GSH enhancing ability.

In the present study, the use of one selected concentration of ALA may not reflect the optimum doses in favour of its ameliorating capacity. Moreover, the study could not provide any clue related to its action at the molecular level. This study was also unable to highlight the status of arsenic accumulation in plasma and erythrocytes, though a clear indication exists regarding the hemato-protective ability of ALA under arsenic insult.

## Conclusion

Based on the experimental data obtained from the rat arsenicosis model, it may be concluded that ALA as a food supplementation has a

beneficial effect against arsenic toxicity. It brings about this protection by restoring near normal hematological, oxidative, and inflammatory status. Further in-depth studies in this direction may help generating knowledge about the defensive role(s) of ALA against arsenic toxicity.

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**Conflict of Interest:** None declared.

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