Indian Journal of Ecology (2025) 52(3): 560-563
DOI: https://doi.org/10.55362/IJE/2025/4536

Manuscript Number: 4536
NAAS Rating: 5.38

Effect of Vitamin C on Arsenic Induced Oxidative Stress in Buffalo Erythrocytes in *Vitro*

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***Abstract*:** Exposure to arsenic, a highly toxic trace metalloid causes disturbances in cellular redox status leading to serious health hazards in dairy animals including buffaloes, subsequently affecting human through consumption of milk. Exposure of buffalo erythrocytes to varying concentration of sodium arsenite (0.01-0.5µg for 2hrs.) decreased the activities of superoxide dismutase, catalase, glutathione peroxidase; and increased the malondialdehyde generation and fragility of erythrocytes in a concentration dependent manner compared to the corresponding controls. Vitamin C treatment @10mg/ml to sodium arsenite (0.5µg) challenged buffalo erythrocytes *in vitro* for 2hrs. effectively modulated the oxidative stress as evidenced from the improved membrane lipid peroxidation, erythrocytic fragility and enhanced activities of superoxide dismutase, catalase and glutathione peroxidase compared to the corresponding controls which can be attributed to the antioxidant characteristics of Vitamin C against arsenic. This study demonstrated that acute exposure of sodium arsenite, trivalent arsenic to the buffalo erythrocytes produces oxidative stress through enhanced membrane lipid peroxidation and depletion of intracellular enzymatic antioxidant defense, which was modulated by vitamin C.

Keywords: Arsenic, Oxidative stress, Buffalo erythrocytes, Vitamin C

Arsenic is widely distributed throughout the environment due to its natural existence and anthropogenic application (Dash et al., 2016). It is one of the ten chemicals of major public health concern listed by the World Health Organization. Contamination of arsenic in water, feed, air and soil, in recent times is of global concern (Dash et al., 2016, Mondal et al., 2021). Environmental arsenic exposure is a serious threat to the livestock health, dairy animals including buffaloes as well as to the human health due to its residual effect and transmission via. buffalo milk consumption, making it one health issue. Both acute/ chronic contamination with arsenic may cause oxidative stress (Hu et al., 2020) which leads to serious health hazards in buffaloes (Dash et al., 2016).

Red blood cells (RBC), essential components of the circulatory system, are particularly susceptible to oxidative damage due to their high oxygen carrying capacity (Duan et al., 2017). Biomolecules in RBC are highly vulnerable to oxidative damage (Guidarelli et al., 2017). Redox-imbalance phenomena caused by arsenic exposure are yet to be understood in buffalo erythrocytes, a major hurdle in the development of therapeutic/ preventive strategies (Dash et al., 2016, Qian et al., 2023). Antioxidant therapy is considered as an efficient primary therapy for arsenic exposure (Mukherjee et al., 2017). Synthetic and chemical based antioxidant therapies are having numerous side effects (Williams et al., 2014, Mukherjee et al., 2017). Thus, nutraceutical antioxidant therapy using Vitamin C can be a choice with no side effects for the improvement of erythrocytic oxidative stress status and health of buffaloes exposed to arsenic (Gracia-Rodriguez and Altamirano-Lozano 2017). The current study investigated, if there is involvement of oxidative stress phenomena in buffalo erythrocytes challenged with arsenic *in vitro* and possible antioxidant effects of vitamin C.

MATERIAL AND METHODS

Preparation of erythrocyte suspension: Adult healthy female buffalo (aged 4.5 years) red blood cells were obtained from Innovative Research Inc. biotechnology company, Novi, Michigan, USA. Packed erythrocytes were washed thrice with phosphate-buffered saline (PBS), pH 7.4 and resuspended in PBS. Erythrocytes were counted using hemocytometer and cell number was adjusted to 2×10^6 cells/ml.

Treatment of erythrocytes with sodium arsenite: Erythrocyte suspensions (2×10^6 cells/ml) were incubated with PBS (Control) or sodium arsenite ($NaAsO_2$) (0.01µg to 0.05 µg) for 2 hrs (acute exposure) at 37 °C as follows to study the effects of arsenic exposure on cellular oxidative stress status.

Control group: Erythrocyte suspension incubated with PBS.
Groups T1, T2, T3, T4 and T5: Erythrocyte suspensions incubated with 0.01, 0.02, 0.03, 0.04 and 0.05 µg $NaAsO_2$ for 2 hrs respectively.

[Maximum Permissible Limit of arsenic in drinking water in view of animal health is 0.01µg/ml (WHO)].

Effect of Vitamin C on sodium arsenite treated buffalo erythrocytes: To study the effects of vitamin C, weighed quantity of Vitamin C was dissolved in phosphate-buffered saline (PBS) for the *in vitro* treatment of sodium arsenite challenged buffalo erythrocytes as follows:

Control group: Erythrocyte suspension incubated with PBS.
Groups T1, T2 and T3: Erythrocyte suspensions incubated with $NaAsO_2$ (0.05 µg) and Vit. C (1, 5 and 10mg/ml respectively) for 2 hrs.

Assay of erythrocyte fragility: Erythrocyte fragility was assayed as per the method of Mrugesh et al., (2011). Briefly, after incubation all the erythrocyte tubes were centrifuged at 1500g for 5 minutes. A tube was prepared by adding 2ml distilled water to 2ml RBC suspension to achieve 100% hemolysis. Absorbance of the supernatant was measured by using UV/VIS spectrophotometer (UV-1601, SHIMADZU) at 540nm. Erythrocyte fragility was expressed as the percentage hemolysis.

Estimation of oxidative stress indices: Malondialdehyde, the product of erythrocyte membrane lipid peroxidation was assayed by the method of Placer et al. (1966) and expressed in nmol MDA per mg. Hb. Superoxide dismutase was estimated according to the method of Nishikimi et al. (1972) and results were expressed as U/mg Hb. Catalase assay was carried out as per the method of Aebi (1984). Activity of Catalase was measured using molar extinction coefficient of hydrogen peroxide and expressed as U/mg Hb. Glutathione peroxidase was estimated as per the method of Flohe and Gunzler (1984). Activity of the enzyme was calculated using molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1} and expressed as U/mg Hb. All estimations were carried out using UV/VIS spectrophotometer (UV-1601, SHIMADZU).

Statistical analysis: Data analysis was carried out by statistical package for social sciences (SPSS) software using independent t-tests.

RESULTS AND DISCUSSION

Effect of sodium arsenite (SA) exposure on oxidative stress status in buffalo erythrocytes *in vitro:* Buffalo erythrocytes induced with sodium arsenite (0.01-0.5µg for 2hrs.) *in vitro* exhibited significantly increased formation of malondialdehydes, an indicator of membrane lipid peroxidation in a concentration dependent manner. Maximum generation of malondialdehyde (17.44 fold increase) was observed at 0.5µg sodium arsenite concentration as compared to the control. Followed by the *In vitro* incubation of buffalo lymphocytes with sodium arsenite (0.01-0.5µg for 2hrs.), initially there is an increased response of the enzymes e.g. superoxide dismutase (T1 and T2), catalase (T1), and glutathione peroxidase (T1) as compared to the corresponding controls. However, with increase in dose of sodium arsenite, the activities of superoxide dismutase (T3-T5), catalase (T2-T5) and glutathione peroxidase (T2-T5) significantly decreased in a concentration dependent manner. This was accompanied by an elevation in the fragility of erythrocytes, an indicator of cell membrane damage in a concentration dependent manner (Table 1).

Effect of vitamin C treatment on sodium arsenite induced oxidative stress in buffalo erythrocytes *in vitro:* *In vitro* co-incubation of sodium arsenite (0.5µg for 2hrs.) treated buffalo lymphocytes with vitamin *C* @ 1mg/ml and 5mg/ml for 2hrs did not affect the membrane lipid peroxidation, activities of catalase and glutathione peroxidase and fragility of erythrocytes. But, good improvement in the activity of erythrocytic superoxide dismutase was observed in group T2 (vitamin C treatment is @ 5mg/ml) which continued to improve in group T3 also (vitamin C @ 10mg/ml). Incubation of lymphocytes with sodium arsenite (0.5µg for 2hrs.) and vitamin C (@10mg/ml) simultaneously for 2hrs significantly improved the membrane lipid peroxidation, activities of catalase, glutathione peroxidase and erythrocyte fragility compared to the corresponding controls (Table 2).

This study, for the first time reported that $NaAsO_2$ exposure increased the membrane lipid peroxidation, as evidenced from the formation of malondialdehyde and

Table 1. Erythrocytic fragility and oxidative stress indices (Mean ± SE) in buffalo erythrocytes treated with sodium arsenite *in vitro*.

Parameters	Control	Group T_1	Group T_2	Group T_3	Group T_4	Group T_5
Erythrocyte fragility (%)	0.02 ±0.003	0.64±0.11	36.35±2.01*	49.18±1.16*	60.40±1.64*	86.36±1.42*
Malondialdehyde (nmol/ mg Hb)	1.63±0.14	2.59 ±0.44	8.77±0.51*	13.37±0.71*	20.61±1.12*	28.44±1.04*
Superoxide dismutase (U/mg Hb)	2.94±0.21	3.88±0.51	10.51±0.72*	1.52±0.13*	1.18±0.20*	0.44±0.03*
Catalase (U/mg Hb)	21.82±1.31	25.16±1.12	17.94±1.03*	14.22±1.32*	9.94±1.08*	4.67±0.98*
Glutathione peroxidase (U/mg Hb)	13.83±1.06	14.08±1.30	10.13±1.11*	7.92±0.81*	5.84±0.57*	2.64±0.33*

*Indicates significant ($p<0.05$) difference from control. Data presented above are of eight independent experiments performed in duplicate

Table 2. Effect of Vitamin C on Erythrocytic fragility and oxidative stress indices (Mean ± SE) in sodium arsenite treated buffalo erythrocytes *in vitro*

Parameters	Control	$NaAsO_2$ treatment group (@ 0.05 µg)	$NaAsO_2$ + Vit. C treatment groups		
			Group T_1	Group T_2	Group T_3
Erythrocyte fragility (%)	0.03±0.001	84.71±1.12*	28.24±1.11*	8.16 ±0.44*	0.22±0.02
Malondialdehyde (nmol / mg. Hb	1.88±0.22	27.08±1.28*	20.18±1.06*	9.53±0.77*	2.51±0.24
Superoxide dismutase (U/mg Hb)	3.31±0.18	0.51±0.06*	1.12±0.04*	4.03±0.33	4.19±0.41
Catalase (U/mg Hb)	22.08±1.60	4.44±0.63*	5.96±0.31*	12.99±1.13*	21.10±1.18
Glutathione peroxidase (U/mg Hb)	14.11±1.31	2.93±0.29*	3.40±0.15*	9.51±0.66*	15.31±1.06

*Indicates significant ($p<0.05$) difference from control. Data presented above are of eight independent experiments performed in duplicate

decreased the level of antioxidant enzymes e.g. superoxide dismutase, catalase and glutathione peroxidase in buffalo erythrocytes in vitro creating oxidative stress environment inside the cell. Arsenic compounds causes imbalance in the redox equilibrium of the cell, thereby causing oxidative stress (Gonzalez-Alfonso et al., 2023). $NaAsO_2$ (trivalent arsenic) can generate reactive oxygen species (ROS) through Fenton reaction which produced toxic effects including apoptosis in different types of animal cells (Mondal and Chattopadhyay 2020). In addition to ROS, formation of peroxyl radicals (ROO), superoxide anion radical (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radical (OH), hydrogen peroxide (H_2O_2) was reported in different animals exposed to arsenic (Nithyashree et al., 2023). Lipid peroxidation, protein oxidation, membrane damage, DNA damage and cell death due to arsenite exposure can be attributed to the generation of hydrogen peroxide, followed by OH radicals (Valko et al., 2016). Trivalent arsenic interacts with sulfhydryl group of biomolecules and may cause lipid peroxidation and protein oxidation even at very lower concentrations in very short duration (Nithyashree et al., 2023). The study also reported the enhanced formation of malondialdehyde in $NaAsO_2$ challenged buffalo erythrocytes which increase the fragility of these cells.

Oxidative damage to cellular biomolecules can be modulated by enzymatic and non-enzymatic antioxidants (Ince et al., 2019). Superoxide dismutase and catalase mutually function in the elimination of ROS. ROS inhibit the activities of antioxidant enzymes leading to alterations in cell's intrinsic antioxidant defense causing disturbed antioxidant/pro-oxidant ratio (Nithyashree et al., 2023). In the present study, lowered superoxide dismutase and catalase activity observed in $NaAsO_2$ treated buffalo erythrocytes can be due to enhanced production of ROS during arsenic metabolism or down-regulation of the synthesis of antioxidant enzymes by the toxic arsenite exposure (Mondal and Chattopadhyay 2020). To combat free radical generation, glutathione peroxidase utilizes reduced glutathione during the course of its action (Yadav et al., 2016). Fragility of erythrocytes was increased in $NaAsO_2$ treated groups which can be attributed to increased lipid peroxidation and decreased enzymatic antioxidant status in the buffalo erythrocytes in this study. Elevated membrane lipid peroxidation with depleted antioxidant status in $NaAsO_2$ treated buffalo erythrocytes cause damage to the membrane lipids, oxidative stress and increases the fragility of these cells.

Vitamin C is a powerful antioxidant with superoxide and hydroxyl radical scavenging activity, as well as metal chelating ability (Gracia-Rodriguez and Altamirano-Lozano 2017). However, its therapeutic effect on oxidative stress due to arsenic exposure in buffalo erythrocytes was unclear (Qian et al., 2023). This study observed that $NaAsO_2$ exposure significantly decreased the intracellular enzymatic antioxidant defense in buffalo erythrocytes which was modulated by vitamin C treatment. Vitamin C also modulated the $NaAsO_2$ induced erythrocytic lipid peroxidation in this study. Oxidative stress due to heavy metals can be neutralized by vitamin C which binds to the metal ions, reduce the catalytic activity and resulting ROS production and prevents their harmful effects (Wang et al., 2022). Vitamin C can be easily absorbed and penetrated through aqueous and membrane environment. It can scavenge free radicals due to its electron deficient double bonds which make it highly reactive towards the free radicals (Williams et al. 2014). The therapeutic effect of Vitamin C in this study is attributed to the above antioxidant characteristics, which successfully modulated the oxidative stress induced by arsenite exposure in buffalo erythrocytes.

CONCLUSION

Buffalo erythrocyte damage due to acute arsenic exposure was mediated via. oxidative stress as evidenced

from the generation of malondialdehyde, the lipid peroxidation product and decreased antioxidant enzymes. The oxidative stress phenomena plays vital role in the process of arsenic exposed erythrocyte damage. Vitamin C @ 10mg/ml effectively modulates the redox imbalance status in buffalo erythrocytes and can be used as an ameliorative measure for improving the antioxidant status and health in dairy buffaloes with environmental arsenic exposure.

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Received 22 February, 2025; Accepted 15 May, 2025