ADDITIVE BENEFICIAL EFFECT OF FOLIC ACID AND VITAMIN B₁₂ CO-ADMINISTRATION ON ARSENIC-INDUCED OXIDATIVE DAMAGE IN CARDIAC TISSUE IN VIVO

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ABSTRACT

Protective effect of folic acid and vitamin B₁₂ supplementation against arsenic-induced alteration of certain biochemical variables indicative of oxidative stress was evaluated in myocardial tissue of male rats of Wistar strain. Treatment with NaAsO₂ at a dose of 6 mg (i.p.)/kg b.w./day for 30 days exhibited a significant reduction in the level of cardiac glutathione content associated with increased malondialdehyde production. The present study also examined that certain antioxidant enzyme activities such as superoxide dismutase, catalase, glutathione-S-transferase, glutathione reductase in cardiac tissues of rats were inhibited following arsenic exposure. In addition, arsenic treatment significantly increased nitric oxide generation in heart of the experimental animals. Oral supplementation of folic acid (50 µg/kg b.w./day) and vitamin B₁₂ (1 µg/kg b.w./day) separately or in combination for the last 14 days of arsenic treatment exhibited differential protective response in reducing the oxidative damage following arsenic treatment. It is also found that during combined exposure of folic acid and vitamin B₁₂, the adverse effects of arsenic were less pronounced in cardiac tissue than their individual effects. This suggests the synergistic beneficial effects of folic acid and vitamin B₁₂ against arsenic-induced oxidative damage in cardiac tissue.

Keywords: Oxidative stress, Sodium arsenite, Cardiac toxicity, Folic acid, Vitamin B₁₂.

INTRODUCTION

Arsenic, being an environmental toxicant, leads to development of serious health hazards affecting human beings. Arsenic mainly via drinking water, food, industrial processes and other sources. Various types of health hazards like acute gastrointestinal symptoms, subacute sequelae resulting in polyneuropathy 5. 7 and chronic symptoms like degenerative, inflammatory and neoplastic changes of skin, and cardiovascular, nervous and reproductive systems are observed in arsenic toxicity 6. 7. Adverse effects of arsenic on tissue metabolic activities through generation of reactive oxygen species (ROS) were reported earlier 5. 6. 7. Reactive oxygen species causes oxidative damage to cellular components including DNA, tissue proteins, fatty acids etc. 8. Arsenic-induced lipid peroxidation through generation of ROS may lead to production of various lipid peroxides like conjugated dienes, alkenes and aldehyde products 9. This abnormal lipid metabolism may be associated with cellular dysfunctions leading to carcinogenesis and cardiovascular diseases 10. Arsenic causes overt endothelial cell injury, cell proliferation and changes in monolayer binding of labeled low-density lipoprotein and permeability of albumin 11, 12. Arsenic is considered as one of the risk factors, associated with cardiovascular diseases 12. It has been established that chronic ingestion of arsenic can lead to cardiovascular disorder with a resulting peripheral vascular disease, atherosclerosis, coronary artery disease, myocardial infarction, ischemic heart disease, and diabetes 13, 14. Saad et al. 15 demonstrated that both arsenic trioxide and imatinib mesilate might have significant cardio-toxic effects. Further studies by Raghu et al. 16 demonstrate abnormal cardiac electrical potential after arsenic trioxide exposure. High-level arsenic exposure is consistently associated with QT prolongation, a risk factor for arrhythmia and sudden cardiac death, reported by Mordukhovich et al. 17.

Various antioxidant vitamins have been shown to have some protective role against arsenic-induced cardiotoxicity. Epidemiological studies have shown that vitamin E is the strongest contributor to the inverse relationship between serum antioxidant concentrations and ischemic heart disease 18. Protective effects of vitamin E on arsenic-induced changes on GSH content, lipid peroxidation level and antioxidant enzyme activity in rat liver were demonstrated by the studies of Ramanathan et al. 19 Folic acid has been shown to reduce the risk of congenital heart defects and cleft lips 20. Folic acid supplements consumed before and during pregnancy may reduce the risk of heart defects in new born baby 21. Recent studies have shown that, vitamin B₁₂ also lowers homocysteine levels and protects against atherosclerosis and other cardiovascular diseases 22, 23. Vitamin B₁₂ also plays a vital role in maintaining methylation reactions that repair DNA and hence prevents cancer 24, 25. The risk of toxicity from folic acid and vitamin B₁₂ is low, because water-soluble vitamins are regularly removed from the body through urine 26. Antioxidant activity was reported to be more efficient when antioxidants were used in combination 27. Thus, inadequate information is available regarding the beneficial effects of folic acid and vitamin B₁₂ in amelioration of arsenic-induced oxidative damage in cardiac tissue, if any. Therefore, the proposed study aims to determine possible influence of arsenic on cardiac antioxidant defense system by which arsenic brings about cardiac damage and also to assess the effects of selective nutritional supplements like folic acid and vitamin B₁₂ individually, or in combination on certain biochemical variables related to oxidative stress markers.

MATERIALS AND METHODS

Materials

Sodium arsenite (NaAsO₂), bovine serum albumin (BSA, 5,5-Dithiobis-2-nitrobenzoic acid (DTNB), reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced glutathione (GSH), trichloroacetic acid (TCA), ethylene diamine tetraacetic acid (EDTA), 1-chloro 2,4-dinitrobenzene (CDNB), Vitamin B₁₂ folic acid, 2-thiobarbituric acid (TBA) and other chemicals used in the study are of analytical grade and were purchased from the Sigma Aldrich, MERCK and SRL.

Animals

Healthy male growing adult albino rats of Wistar strain, weighing between 140 gm to 150 gm were acclimatized under laboratory conditions for two weeks before starting the experiment. They were provided with standard protein diet (18% casein diet) 5 and supplied with drinking water ad libitum. They were kept in animal house by maintaining standard conditions of temperature (22°C to 25°C) and humidity (50%) with alternating 12 hours light/dark cycle.

Experimental design

For the present study 30 male albino rats of Wistar strain were taken. They were divided into five different groups of equal average body weight and kept in well ventilated cages. The Institutional animal ethical committee approved the design of the experiment. They were labeled namely Group I, Group II, Group III, Group IV and Group V. The dose of arsenic has been selected from the report of Pal...
and Chatterjee. The treatment schedule for the present study is given below:-

Group I: This group was regarded as control group and given 0.9% NaCl/day (i.p.) for 30 days.

Group II: This group was considered as arsenic-treated group and 6 mg of NaAsO$_2$/kg b.w. /day (i.p.) was given for a period of 30 days.

Group III: Animals of this group were treated with 6 mg of NaAsO$_2$/kg b.w./day (i.p.) for 30 days followed by folic acid supplementation at a dose of 50μg/kg b.w./day (orally) for last 14 days of arsenic treatment.

Group IV: This group was treated with NaAsO$_2$ at a dose of 6 mg /kg b.w./day (i.p.) for 30 days and oral administration of vitamin B$_12$ was given at a dose of 0.65μg/kg b.w./day for the last 14 days of arsenic treatment.

Group V: This group was treated with 6 mg of NaAsO$_2$/kg b.w./day (i.p.) for 30 days followed by co-administration with folic acid and vitamin B$_12$ at the same dose and duration mentioned earlier.

Animal sacrifice and heart tissue collection
After animal treatment was over, rats were sacrificed by cervical dislocation following ether anesthesia according to the guidelines proposed by the Institutional Animal Ethical Committee for the protection of Control and Supervision on Animal Experiments (CPCSEA), Ministry of Environment and Forests, Government of India. Hearts from the experimental animals were quickly excised and washed in ice-cold saline, blotted dry and kept at -20°C until analysis.

Preparation of tissue homogenate
A 5% tissue homogenate of cardiac tissue was prepared in 0.1 M potassium phosphate buffer (pH 7.4) using all glass homogenizer and kept frozen at -20°C until biochemical analysis was performed.

Biochemical analysis

Reduced glutathione (GSH) content
The GSH content in heart was estimated by the method of Ellman as modified according to Davila et al. The 5% tissue homogenate was treated with 20% TCA containing 1 mM EDTA to allow precipitation of proteins. The centrifugate was then treated with Ellman’s reagent (DTNB in 1% sodium citrate). The reading was taken in a spectrophotometer at 412 nm. Tissue glutathione level was calculated from the standard curve generated using aliquots of solution having known concentration of GSH.

Tissue lipid peroxidation (LPO) level
LPO level in the homogenates of heart was determined according to the method of Buege and Aust. One millimolar EDTA was used in the reaction mixture to chelate iron and reduce its interference in the peroxidation reaction of unsaturated fatty acids. The reaction mixture was then heated at 80°C for 5 minutes. The optimal density was read at 533 nm. The molar extinction coefficient, 1.56 x 10$^3$ cm$^{-1}$/mmol of malondialdehyde was used to calculate the malondialdehyde production.

Nitric oxide (NO) production
Nitric oxide level in heart was measured by the method of Rasool et al. The 5% tissue homogenate was prepared in 0.25 M sucrose solution and centrifuged at 6000 rpm at 4°C for 10 minutes. Equal volume of 0.25 M sucrose solution, 1% sulfanilamide and 0.1% naphthylethylene diamine hydrochloride were then added to the supernatant and mixed well. After 20 minutes of the total reaction, the optimal density was read at 550 nm in a spectrophotometer.

Glutathione reductase (GR) activity of cardiac tissue
The 10,000 g supernatant of 5% tissue homogenate was used for estimation of GR activity by the method of Carlberg and Mannervik. The assay system consisted of phosphate buffer (0.2M, pH 7.0, containing 2mM EDTA), 20 mM GSSG, 2 mM NADPH and supernatant. The enzyme activity was quantitated at 25°C by measuring the disappearance of NADPH at 340 nm. The enzyme activity was expressed as nmol of NADPH oxidized per minute per mg of protein.

Glutathione S-transferase (GST) activity
Glutathione S-transferase activity will be assayed by the method of Warholm et al. 0.01M (pH 6.5) phosphate buffer containing 1 mM EDTA, 20 mM GSH and 20 mM CDNB were added to the supernatant in a cuvette and the increase in absorbance was noted at 340 nm. The activity of GST was expressed as nmole of GSH-CDNB conjugate formed/min/mg of protein.

Catalase activity of cardiac tissue
A 5% tissue homogenate was prepared with 0.1M phosphate buffer (pH 7.4) with 1% triton X-100. The catalase activity was measured by calculating the rate of degradation of H$_2$O$_2$ the substrate of the enzyme. The enzyme activity was expressed as μmole H$_2$O$_2$ utilized per minute per mg of protein.

Superoxide dismutase (SOD) activity of cardiac tissue
The 10,000 g supernatant of 5% tissue homogenate was used for estimation of SOD activity by the method of Martin et al. The assay of SOD activity is based on the SOD-mediated increase in the rate of auto-oxidation of hematoxylin in aqueous alkaline solution, which yields a chromophore with maximum absorbance at 560 nm. The enzyme activity was expressed as units per minute per mg of protein.

Tissue protein content
Tissue protein was estimated by the method of Lowry et al. using bovine serum albumin as the standard protein.

Statistical analysis
The values were expressed as mean ± SEM. The data were statistically analysed by one way ANOVA to find whether scores of different groups differ significantly. It was then followed by multiple comparison t-test using Statistica software (version 9) to determine the significance of differences between the two related groups. The p < 0.05 was considered statistically significant.

RESULTS
Table 1 represents the changes in tissue GSH content, LPO level and NO generation following arsenic treatment with or without vitamin B$_12$ and folic acid supplementation. The study reveals that GSH content decreased (p<0.001) by 48.7% in cardiac tissue following exposure to arsenic at the present dose and duration. Vitamin B$_12$ supplementation in arsenic-treated rats restored the reduced GSH content of heart by 67.2% from the arsenic-treated group (p<0.001). Similarly, folic acid exhibits some partial protective effects against arsenic-induced alteration of cardiac GSH content (43.6% restoration, p<0.001). Exposure of folic acid in combination with vitamin B$_12$ shows additive effect in restoration of depleted GSH content by 113.8% (p<0.001). Change in LPO level (Table 1) shows that arsenic treatment increased LPO level in cardiac tissue by 58.5% (p<0.001). Vitamin B$_12$ supplementation alone was found to appreciably check the increased cardiac LPO level due to arsenic treatment. The counteraction was found to be 52.7%, when compared with arsenic treated group (p<0.001). Folic acid supplementation alone also counteracted (37.8% restoration, p<0.001) the elevated LPO level following arsenic exposure. Combined treatment of folic acid and vitamin B$_12$ has much pronounced effect in reduction of cardiac LPO level even below the control value. The restoration was found to be 65.3% compared with arsenic-treated group (p<0.001).

Table 1 further demonstrates that the change in NO level in arsenic-exposed animals was appreciably counteracted by folic acid and vitamin B$_12$ supplementation when administered conjointly. The study reveals that the tissue level of NO was increased (p<0.001) by 49.56% following arsenic treatment. Folic acid alone restored the increased level of NO by 22.7% (p<0.001), whereas vitamin B$_12$
restores NO level by 66.1% (p<0.001), which indicates vitamin B₁₂ has better ameliorative effect than folic acid in restoration of NO level in arsenic exposed rats. Combined supplementation of vitamin B₁₂ and folic acid was found to restore the enhanced NO level by 57.2% (p<0.001) from the arsenic treated animals.

Table 1: Changes in GSH content, LPO, NO level, GR and GST activities in cardiac tissue due to the exposure of arsenic with or without folic acid and vitamin B₁₂ supplementation

<table>
<thead>
<tr>
<th>GROUPS OF ANIMALS</th>
<th>GSH (μM/mg protein)</th>
<th>LPO (nmoles MDA/mg protein)</th>
<th>NO (μM/mg protein)</th>
<th>GR (nM/min/mg protein)</th>
<th>GST (nM/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (6)</td>
<td>35.83 ± 1.26</td>
<td>1.4 ± 0.02</td>
<td>1.16 ± 0.02</td>
<td>90.78 ± 0.77</td>
<td>3.24 ± 0.07</td>
</tr>
<tr>
<td>As-treated (6)</td>
<td>18.37 ± 0.75</td>
<td>2.3 ± 0.04</td>
<td>2.3 ± 0.04</td>
<td>44.14 ± 0.54</td>
<td>1.59 ± 0.01</td>
</tr>
<tr>
<td>As-treated + folic acid supplemented (6)</td>
<td>26.39 ± 0.71</td>
<td>1.88 ± 0.04</td>
<td>54.23 ± 0.54</td>
<td>2.14 ± 0.01</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>As-treated + vitamin B₁₂ supplemented (6)</td>
<td>30.72 ± 0.68</td>
<td>0.78 ± 0.03</td>
<td>67.41 ± 0.70</td>
<td>2.74 ± 0.02</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>As-treated + folic acid + vitamin B₁₂ supplemented (6)</td>
<td>39.28 ± 1.14</td>
<td>0.77 ± 0.03</td>
<td>0.985 ± 0.03</td>
<td>83.36 ± 0.86</td>
<td>3.37 ± 0.02</td>
</tr>
</tbody>
</table>

Values are Mean±S.E.M.
Figures in the parentheses indicate the number of animals.
p<0.05 compared with control group.
p<0.001 compared with arsenic treated group.

Figure 1 demonstrates the effect of folic acid and vitamin B₁₂ supplementation on arsenic-induced changes in SOD activity in cardiac tissue. The SOD activity in cardiac tissue was decreased significantly by 38.5% (p<0.001) after arsenic treatment at the present dose and duration. Folic acid alone results in 20.8% restoration (p<0.001) of cardiac SOD activity in arsenic exposed rats, whereas vitamin B₁₂ restored the enzyme activity by 35.2% (p<0.001). Conjoint administration of folic acid and vitamin B₁₂ appreciably prevented arsenic-induced change in SOD activity in cardiac tissue. The restoration was found to be 66.8% (p<0.001).

Fig 1: Effect of folic acid and vitamin B₁₂ supplementation on arsenic-induced change in cardiac SOD activity

Values are Means±SEM, p<0.001 compared with control group and p<0.001 compared with arsenic treated group.

DISCUSSION

The beneficial effect of combined supplementation of two nutritional elements namely folic acid and vitamin B₁₂ on arsenic-induced alteration of antioxidant defense system was evaluated in cardiac tissue of rat. Arsenic treatment at the present dose and duration decreased the GSH content in cardiac tissue (Table 1). Glutathione exhibits significant role in the detoxification of arsenic because GSH...
is required as a co-factor for the optimal activity of methionine adenosyltransferase which facilitates methylation of arsenic. Depletion of tissue glutathione level has been found to be a causative factor in arsenic-induced oxidative damage. It has been established by the observation of Rana et al. that arsenite binds with nucleophilic sulphydryl groups and thereby reducing GSH content in tissue, aggravating the oxidative threat to tissue. Perturbation of glutathione content in cardiac tissue was reported earlier. They also reported that short-term arsenic toxicity in rats produces a significant decrease in cardiac GSH concentration associated with increased lipid peroxidation level. An inverse correlation between arsenic-induced changes in lipid peroxidation and GSH content in heart was observed in earlier studies. These findings on heart are also supported by our present study, where cardiac GSH depletion was associated with increased lipid peroxidation in that tissue. Cytotoxic effects of lipid peroxidation have been established. Overproduction of lipid peroxide causes destabilization in cellular lipid substances, especially membrane structures. The decreased glutathione reductase activity in the heart after arsenic exposure (Table 1) may be a contributory factor for diminished GSH concentration in cardiac tissue in addition to increased utilization of GSH for neutralizing free radicals generated by arsenic treatment.

The present study further reveals that nitric oxide concentration in cardiac tissues was significantly increased in arsenic exposed rats (Table 1). Nitric oxide serves as an important cellular signaling molecule in many physiological and pathological processes in mammals including humans. High levels of nitric oxide are deleterious for organs including heart. It is assumed that high level of NO may be responsible for generation of peroxynitrite anion via peroxidative stress caused by arsenic. Arsenic toxicity is tissue specific that means toxic effects of arsenic in different tissues vary according to their detoxification capability, dose and duration of the exposure of arsenic as well as tissues individual response to oxidative stress caused by arsenic. This may be responsible for variation in the results obtained in a specific tissue with others when those tissues are exposed to arsenic even at similar dose and duration.

The present study also demonstrates that exposure to arsenic significantly decreased the SOD activity in cardiac tissue (Figure 1). This finding suggests that there was an excess accumulation of superoxide anion in this tissue after arsenic exposure, which may be responsible for increased free radical formation in cardiac tissue. Studies by Lynn et al. and Matsui et al. indicated that arsenic-induced cytotoxicity is mediated through generation of reactive oxygen species (ROS), such as free hydroxyl radical, superoxide anion, hydrogen peroxide, etc. The production of free radicals due to arsenic exposure depends on the dose and duration of exposure as well as the detoxifying capacity of cells against arsenic-induced oxidative stress. Arsenic treatment in our present experiment decreased the catalase activity in heart (Figure 2). Earlier few reports have been suggested that arsenic-induced decreased cardiac catalase activity may be due to its adverse effects on cellular antioxidant defense system. Consequently, over accumulation of H₂O₂ in cardiac tissue of arsenic-exposed animals may enhance the free radical production and promotes arsenic-induced oxidative damage. The activity of glutathione S-transferase was significantly inhibited by arsenic exposure at the present dose and duration (Table 1). As glutathione S-transferase activity detoxifies endogenous compounds such as peroxidised lipids, xenobiotics etc.; so decreased GST activity indicates overaccumulation of lipid peroxides in cardiac tissue leading to oxidative stress. The present observation is also supported by the earlier studies of Maiti and Chatterjee and Manna et al., where alteration of antioxidant enzyme activities was proposed to be involved in arsenic-induced cardiac tissue damage.

Our present study further reveals that folic acid and vitamin B₁₂ supplementation in arsenic-treated rats appreciably counteracted the changes in cardiac GSH contents, lipid peroxide levels and nitric oxide level (Table 1). Vitamin B₁₂ supplementation alone in arsenic-treated rats restored the reduced GSH content of heart by 67.2% (p<0.001), whereas folic acid has some partial protective effects against arsenic-induced alteration of cardiac GSH content (43.6% restoration) (p<0.001). Synergistic beneficial effects were found in restoration of cardiac GSH content when folic acid and vitamin B₁₂ were administered conjointly. It has been reported earlier that folic acid consumed before and during pregnancy may reduce the risk of heart defects in the newborn baby. Earlier studies have shown that, vitamin B₁₂ also lowers homocysteine levels and protects against atherosclerosis and other cardiovascula diseases. Vitamin B₁₂ plays a vital role in maintaining methylation reactions that repair DNA and hence prevents cancer. It has already been established that arsenic is detoxified through methylation reaction involving S-adenosylmethionine and the enzyme methytransferase. Folic acid and vitamin B₁₂ both help to increase endogenous methionine because vitamin B₁₂ acts as a cofactor for the enzyme methionine synthase which catalyzes the synthesis of endogenous methionine from S-adenosylmethionine. Methionine synthase has significant role in folic acid metabolism and maintains folic acid pool in the body. Folic acid also aids endogenous methionine level, as 5-methyltetrahydrofolate (circulating folate) is the methyl group donor in the methylation reaction to convert homocysteine to methionine. As already mentioned that arsenic is detoxified in human body through methylation process and methionine plays an important role in this, it is suggested that vitamin B₁₂ and folic acid promote detoxification of arsenic and thereby reducing the oxidative stress caused by arsenic. Previous studies by Pal and Chatterjee reported that dietary supplementation of methionine has significant ameliorative effect against arsenic-induced alteration of metabolic toxicity. These findings suggest, therefore, that folic acid and vitamin B₁₂ may serve as antioxidant agents towards reducing their ability to reduce excess accumulation of arsenic in cardiac tissue.

In the present study the lipid peroxidation is clearly reflected in increased MDA level in heart in response to arsenic toxicity. The present observation further reveals that the LPO level is reversed completely in both folic acid and vitamin B₁₂ supplemented groups. Their combined administration was found to be stronger than their individual effects in reducing lipid peroxide end product level in cardiac tissue. These results suggest that supplementation with folic acid and vitamin B₁₂ may be predicted as a possible nutritional management strategy against arsenic-induced cardiotoxicity. The present study further reveals that arsenic-induced increased NO content was appreciably counteracted by vitamin B₁₂ and folic acid supplementation. These observations further substantiate synergistic protective effect of vitamin B₁₂ and folic acid in arsenic-induced oxidative stress in heart. In addition to above parameters vitamin B₁₂ and folic acid supplementation reversed the arsenic-induced changes in antioxidant enzyme activities like SOD, catalase, glutathione reductase and glutathione S-transferase in heart.

From the present investigation, it is thus suggested that supplementation of either vitamin B₁₂ or folic acid or their combination has differential antioxidative effects against arsenic-induced oxidative stress in cardiac tissue of rats. It is further revealed that vitamin B₁₂ and folic acid synergistically exhibit better protective effect in restoration of almost all of the parameters of antioxidant defense system studied in cardiac tissue of arsenic-treated animals. It is also suggested that vitamin B₁₂ or folic acid reduces arsenic-induced cardiotoxicity possibly through detoxification of arsenic by enhancing methylation reaction.

CONCLUSION

From the present study, it is concluded that subchronic exposure of arsenic alters certain cellular antioxidant (GSH) and antioxidant enzymes (SOD, CAT, GR and GST), leading to overproduction of harmful metabolites like manoldialdehyde and nitric oxide in cardiac tissue. Folic acid and vitamin B₁₂ co-administration counteracted or eliminated most of the changes in antioxidants and antioxidant enzymes due to exposure to arsenic. It is thus further concluded that conjoint supplementation of folic acid and vitamin B₁₂ following subchronic exposure to arsenic decreases free radical-mediated cytotoxicity by restoring the cellular antioxidant status, and accordingly may serve as a prospective protective agent against arsenic-induced oxidative stress in cardiac tissue.
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CONFLICT OF INTEREST

All the authors declare that they have no conflict of interest.

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