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Abrogation of sodium arsenite driven uterine antioxidant exhaustion and tissue impairment: Role of B₁₂ and folate

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Abstract

Aim: Rapid emergence of arsenic pollution demands the development of novel adjunct with non-invasive painless oral therapeutic agent to combat against arsenic associated health hazards by replacing conventional painful chelating therapy. In the present study, the modulatory effects of vitamin B₁₂ and folic acid (folate) in the amelioration of arsenic mediated uterine disorders were examined.

Methodology: In this experimental study, Wistar strain adult female rats were fed sodium arsenite (As³⁺) contaminated water (0.4 ppm) in conjunction with vitamin B₁₂ (0.04 µg and 0.07 µg) plus folate (2.0 and 4.0 µg) alone and or in combination respectively per 100 g b.wt. per day for seven estrous cycles (28 days).

Results: Rats those underwent arsenic exposure showed significant impairments in the status of uterine antioxidant profile as evident from enzymatic assay and electrozymogram study of superoxide dismutase, catalase and peroxidase with an abnormal increase in conjugated diene and malondialdehyde levels. Mutagenic uterine DNA-breakage and tissue damages were prominent following As³⁺ consumption by the rats. All impairments were totally or partially attenuated by the co-treatment with these two B vitamins in arsenic exposed rats.

Interpretation: The mechanisms might be coupled with the enhancement of antioxidant defense system, partly through the elimination of arsenic with the involvement of S-adenosine methionine pool (SAM) since, vitamin B₁₂ and folic acid are two major regulators of this pool.

Introduction

Drinking arsenic contaminated water creates abrupt threats to the public health and future prospects of agriculture and industries in the affected areas in several countries. The World Health Organization has set a provisional guideline for a tolerable limit of 0.01 mg l^{-1} arsenic in drinking water, whereas in Asian countries like Bangladesh and India the level is 40 times higher than the recommended safe limit (Chattopadhyay *et al.*, 2002). Arsenic imposes its unfavorable effect on fetal growth directly and may also inhibit the transportation of nutrient to the fetus by damaging the placental tissue via oxidative stress (Rahman *et al.*, 2009). Long term arsenic exposure can cause male and female reproductive hazards with alteration of ovarian and testicular steroidogenesis, disruption of ovarian and testicular tissue, arrestation of spermatogenesis and folliculogenesis etc. Sodium arsenite can suppress the activity of hypothalamico-pituitary-gonadal axis and resist the natural folliculogenesis by inhibiting the plasma FSH and LH levels (Chattopadhyay and Ghosh, 2010). The adverse effect is also noted on hypothalamico-pituitary-adrenal axis in arsenic affected rats due to low level of gonadal steroidogenesis. Arsenic increases the ACTH from the pituitary that sustains the hypertrophy of the adrenal gland and elevates the synthesis of Δ^5 , 3β -HSD (hydroxysteroid dehydrogenase), plasma corticosterone (Chattopadhyay *et al.*, 2003). Arsenic mediated reproductive hazards may be due to the disturbance in signaling pathway of the steroid hormone (Nakareangrit *et al.*, 2015) by the regulation of gene expression and not by the receptor itself (Davey *et al.*, 2007). Arsenic induces lesions in the rat uterus and is subsequently primed to reproductive failures by mimicking an estrogenic mechanism (Stoica *et al.*, 2000). Arsenic treatment substantially down regulates the expression of vascular endothelial growth factor (VEGF) in the uterus and estrogen receptor alpha. Arsenic may suppress the action of estradiol altering cell cycle regulatory proteins like CDK4 and cyclin D1 (Chatterjee and Chatterji, 2010). Inside cells, reactive oxygen species (ROS) are formed during metabolic processing of the arsenicals and results in tissue injury. One-carbon (1C) metabolism is important in the methylation of arsenic via S-adenosylmethionine (SAM). Folic acid (FA) and cobalamin (B_{12}) are involved in this process by regulating the status of homocysteinemia internally in response to arsenic exposure (Gamble *et al.*, 2006). Earlier, B_{12} and folic acid has been reported to act as synergistic safeguards in hepatic arsenic detoxification (Chattopadhyay *et al.*, 2012), but reproductive benefits have yet to be documented. Very often it is seen that detoxification of toxin from the liver is not essentially sufficient to protect other organs. Arsenic exposed female population often suffers from infertility (Chattopadhyay *et al.*, 2003). The available drug British Anti Lewsite (BAL) against arsenic manifests with moderate to severe side effects. Thus, the aim of this study was to elucidate the role of non-invasive oral application of B_{12} and folic acid in the amelioration of arsenic mediated uterine degeneration.

Materials and Methods

The study was conducted with the approval of our institution's ethics committee (ethical clearance item no. 3. Vile, 25.09.12), following the standards of Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment, Forest and Climate Change, Government of India. Forty eight female albino Wistar strain rats of 60 days age, weighing $135 \pm 5 \text{ g}$ were selected in this experiment and maintained under standard laboratory condition 12 hr L: 12 hr D and $30 \pm 2^\circ\text{C}$ temperature with free access to food (Standard Laboratory Diet) and water *ad libitum*. The estrous cycle of all rats was synchronized by oral dose of estradiol of $1.0 \mu\text{g } 100 \text{ g}^{-1} \text{ b. wt.}$. The experiment was started on the same day when all the rats showed synchronization in their estrous cycle. Drinking water contaminated with sodium arsenite (As^{3+}) with 0.4-ppm and administered in rat per 100 g b.wt. per day for 28 days. Vitamin B_{12} and Folic acid were supplied through gavages. Animals were sacrificed on day twenty nine. Serum was separated and organs were dissected out for biochemical and histological examinations and preserved at -20°C in several aliquots.

Rats were divided equally as follows:

Group I: Vehicle treated control; **Group II:** As^{3+} (0.4-ppm per 100 g b.wt. per day); **Group III:** As^{3+} (0.4-ppm per 100 g b.wt. per day) + B_{12} (0.04 μg per 100 g b.wt. per day); **Group IV:** As^{3+} (0.4-ppm per 100 g b.wt. per day) + FA (2.0 μg per 100 g b.wt. per day); **Group V:** As^{3+} (0.4-ppm per 100 g b.wt. per day) + B_{12} (0.04 μg per 100 g b.wt. per day) + FA (2.0 μg per 100 g body wt. per day); **Group VI:** As^{3+} (0.4-ppm per 100 g b.wt. per day) + Vit- B_{12} (0.07 μg per 100 g b.wt. per day); **Group VII:** As^{3+} (0.4-ppm per 100 g b.wt. per day) + FA (4.0 μg per 100 g b.wt. per day); **Group VIII:** As^{3+} (0.4-ppm per 100 g b.wt. per day) + Vit- B_{12} (0.07 μg per 100 g b.wt. per day) + FA (4.0 μg per 100 g b.wt. per day).

Estimation of malondialdehyde and conjugated dienes levels : Uterine tissue was homogenized (20% w/v) in ice-cold phosphate buffer (0.1 mol l^{-1} , pH 7.4). The supernatant was collected by centrifugation at $15,000 \times g$ at 4°C for 3 min for the estimation of malondialdehyde (MDA) and conjugated dienes (CD). The MDA was determined from the reaction of thiobarbituric acid with MDA. The amount of MDA formed was measured by taking the absorbance at 530 nm ($\epsilon = 1.56 \times 10^5 \text{ mol}^{-1} \text{ cm}^{-1}$) (Jendryczko and Drózd, 1988). The CD was determined by a standard method, where lipids were extracted with chloroform-methanol (2:1) and centrifuged at $1,000 \times g$ for 5 min. The lipid residue was dissolved in 1.5 ml of cyclohexane, and finally, the amount of hydroperoxide formed was measured at 233 nm (Okhawa *et al.*, 1989).

Spectrophotometric assay of super oxide dismutase (SOD) and catalase activities : Uterine tissue was homogenized in 100 mM l^{-1} chilled Tris-HCl buffer containing 0.16 mol l^{-1} KCl (pH 7.4) to

give a tissue concentration of 10% (w/v) and centrifuged at 10,000 × g for 20 min at 4°C. The reaction mixture was prepared by mixing 800 µl of TDB (Merck), 40 µl of 7.5 mM I⁻¹ NADPH (Sigma), 25 µl of EDTA-MnCl₂ and 100 µl of the tissue supernatant. The activity of SOD in this mixture was monitored at 340 nm from the rate of oxidation of NADPH (Paoletti *et al.*, 1990).

Catalase activity was assayed spectrophotometrically using same homogenate (Sinha, 1972). The reaction was stopped at different time intervals by the addition of a dichromate-acetic acid mixture and H₂O₂ and was determined as chromic acetate. One unit of catalase activity was expressed as a mole of H₂O₂ consumed min⁻¹ mg⁻¹ protein.

Assessment of SOD and catalase by native gel electrophoresis : Uterine horns were homogenized (20% w/v) in ice cold PBS (1M, pH 7.4) centrifuged at 10,000 × g for 20 min at 4°C. A 12% SOD activity gel assay system was used for electrozymographic imprint. The SOD active site developed a clear area of achromatic bands competing with nitroblue tetrazolium for O₂⁻ radical (Christine *et al.*, 2010). The gel was scanned when maximum contrast between the band and background was achieved.

A 50 µg protein per sample was electrophoresed on 8% PAGE for catalase activity. Gels were kept at 0.003% H₂O₂ solution for 10 min and then placed in the staining mixture which contained 2% potassium ferricyanide and 2% ferric chloride. Bluish yellow bands were appearing against a blue green background.

Assessment of uterine peroxidase activity and electrozymogram of peroxidase : Uterine tissues were homogenized in 0.1 M of phosphate buffer solution (pH 7.0) and centrifuged at 18,000 × g for 15 min at 5°C (Sadasivan and Manickam, 1966) for enzymatic assay of peroxidase. A cocktail of 20 Mm of guaiacol and 0.1 ml of supernatant was read in the presence of 0.3 ml of H₂O₂ (12.3 mM) as substrate. The time was noted when the absorbance increased (436 nm) by 0.1 unit.

For the electrozymogram study of uterine peroxidase uterine horns were homogenized (20%;w/v) in ice cold PBS (0.1M pH 7.4) and centrifuged at 10,000 × g for 20 min at 4°C and extracted protein was loaded at 8% native gel. Peroxidase zymogram was developed by benzidine in 30% H₂O₂ solution till the appearance of brown colour (Hathama and Nuha, 2014).

Assessment of total serum Lactate Dehydrogenase (LDH) : Using Tulip Assay kit from Tulip India Group, total serum LDH activity was assayed from the decrease in absorbance of oxidation of NADH to NAD. This decrease in absorbance was proportional to the LDH activity in the sample.

For an electrozymographic study of the enzyme agarose gel of 1.2% in 50 mM Tris-HCl buffer pH 8.2 was electrophoresed

with 20 µl serum at 170 V. Agarose gel was developed with slight modification in the presence of H₂O, 1.0 M Tris, tetrazolium-blue, phenazine-methosulphate, Na-lactate and NAD and then incubated at 37°C to develop color reaction for 30 min following the rinsing of the gels with water and observed under light exposure (Brandt *et al.*, 1987).

DNA analysis : Uterine horns were used for DNA preparation and the cell pellet was treated with 500 µl lysis buffer (50 mM Tris, pH 8.0, 20 mM EDTA, 10 mM NaCl, 1% SDS, 0.5 mg ml⁻¹ proteinase K) for 15 min at 4°C and centrifuged in chilled conditions at 12,000×rpm for 20 min. The supernatant was collected and treated with 1:1 mixture of phenol: chloroform with gentle agitation for 5 min and precipitated in two parts of cold ethanol and one tenth part of sodium acetate (Garcia-Martinez *et al.*, 1993). After spinning down and decantation, the precipitate was resuspended in 30 µl of deionized water-RNase solution (0.4 ml water + 5 µl of RNase) at 37°C. The 8.0 % agarose gel with ethidium bromide was run at 65 V and documented in gel documentation system.

Comet assay : Comet assay was performed following the method of Singh *et al.*, (1988). To a 25 ml of cell suspension (105 cells) low melting point agarose (0.6%) was added in PBS at 37°C and electrophoresed. Following the completion of electrophoresis for 30 min at 25 V and 300 mA, slides were then neutralized with PBS and stained with a solution of 10 mg ml⁻¹ ethidium bromide for 5 min. Washing with water, excess stain was removed. Slides were analyzed using a fluorescence microscope (Nikon, Eclipse LV100 POL), with the VisComet (ImpulsBildanalyse) software.

Histopathological assessment of uterus : The uterine horns were embedded in paraffin laterally, sectioned at 5 µM by a semi-automated microtome (Leica Biosystems). Sections were stained with eosin and hematoxylin (Harris) and observed under a microscope (Nikon, Eclipse LV100, magnification ×400) to evaluate the histological changes. Fractalyse 2.4 software was used to analyze the surface morphology and fractal dimension of uterus from its histological image to assess possible fibrotic status.

Densitometry and statistical analysis : UN-SCAN-IT gel 7.1 software was used to measure the band intensity with respect to the control band (100%). Data from the arsenic ingested group or B vitamins co-administered group were compared with control by utilizing ANOVA, followed by multiple comparison two tailed t-test.

Results and Discussion

The current results suggest that trivalent form of arsenic yields reactive oxygen species in association with H₂O₂, and thus forms several lipid peroxides and conjugated diene as the end products. A significant elevation was observed in the uterine MDA

(323%) and CD (129%) level in the arsenite-induced group compared to the control group (Fig. 1). Regarding SOD inhibition, it may be clarified that uterine tissue may face an increased production of superoxide anion (O_2^-) in response to arsenic ingestion as a consequence of diminished conversion of superoxide anion radical to H_2O_2 in uterine tissue. Henceforth, this enzymatic exhaustion, as shown electrozymographically, indicates a generation of free radicals in uterus during arsenic metabolism. (Fig. 2).

In the present study, a significant diminution in uterine SOD (200%), catalase (180%) and peroxidase (316%) activities were observed (Fig. 2A, Fig. 3A) in arsenic-exposed versus control group when spectrophotometric determination was performed for these enzymes. Moreover, electrozymogram shown in Fig. 2B, 2C and 3C revealed that the electrophoretic intensity of these three enzymatic bands was reduced in arsenic ingested group significantly in comparison to vehicle treated control. Reduced antioxidative enzymatic activities were addressed for cell cycle arrest (2/M phase),

necrosis and apoptosis in the male reproductive organ (Fang et al., 2013).

In the present study, arsenic ingestion induced uterine DNA laddering and smearing (Fig. 5A); and single cell DNA damage due to excess free radicals (Fig. 5B). This incidence may promote the induction of necrotic and apoptotic changes in uterine tissue (Ciaccio et al., 1998). The formation of ROS may finally lead to DNA damage by trivalent arsenicals and also initiating a suppression of DNA repair systems as well as repair of oxidative DNA damage. Arsenic (III) may influence hypomethylation of uterine DNA in expense of a well-known methyl donor of arsenic metabolism S-adenosyl methionine (SAM). Necrotic and apoptotic tissue death along with extreme DNA damage were validated by the arsenicals and its methylated derivative and this might be further influenced by the alteration in SOD activity (Kligerman et al., 2003; Salnikow and Zhitkovich, 2008).

LDH is also designated to be a cancer-specific biomarker and is not generally increased in patients without

Fig. 1 : Protective effect (dose dependent) on malondialdehyde (MDA) and conjugated dienes (CD) in uterine tissue by co-administration of Vitamin B₁₂ and folic acid in As³⁺ intoxicated rats. Comparatively higher doses of vitamin B₁₂ (0.07 µg) and folic acid (4.0 µg) alone or in combination were more effective for the above protection. Each bar represents mean of six replicates, ANOVA followed by two-tailed Student's t test were used *p<0.05; **p<0.01

Table 1 : Dose dependent changes in general body growth and uterine-somatic index of rat is shown in response to sodium arsenite ingestion and its protection of uterine weight by vitamin B₁₂ and folate though As³⁺ has no significant impact on the general growth status of the animal

Mode of treatment	Initial body wt. (g)	Final body wt. (g)	Uterine-somatic index (mg%)
Vehicle treated control	137±3.0	157±2.4	139.3±7.0
As ³⁺ (0.4ppm)	139±3.8	148±2.5	109±4.5**
As ³⁺ (0.4ppm)+ Vit-B ₁₂ (0.04 µg)	138±3.5	149±2.2	112±5.0**
As ³⁺ (0.4ppm)+ Folic acid (2.0 µg)	137±3.2	148±2.0	111.5±5.5**
As ³⁺ (0.4ppm)+Vit-B ₁₂ (0.04 µg) + Folic acid (2 µg)	139±3.5	150±3.0	115.2±6.3**
As ³⁺ (0.4ppm)+ Vit-B ₁₂ (0.07 µg)	138±2.9	150±3.0	120.5±6.8*
As ³⁺ (0.4ppm)+ Folic acid (4.0 µg)	139±2.8	151±2.8	122.3±6.8*
As ³⁺ (0.4ppm)+Vit-B ₁₂ (0.07 µg)+ Folic acid (4.0 µg)	138±3.0	154±2.5	134.5±5.5

Data represent here is mean. Data of As³⁺ -ingested or vitamins co-administered groups are compared to the corresponding vehicle treated group (Student's t test) *p<0.05; **p<0.01

Fig. 2 : SOD and Catalase activity show differential pattern in uterine tissue of female albino rats. (A) SOD and Catalase activity measured in cell free extracts. Higher doses of vitamin B₁₂ (0.07 µg) and folic acid (4.0 µg) alone or in combination in cell free extract depicted more pronounced protection of SOD and Catalase activities; (B) tissue extracts from rats (co-treated with these above, comparatively higher doses of these two B vitamins) containing protein in each lane was electrophoresed for obtaining SOD and Catalase bands and (C) Densitometric analysis of the above bands. Each bar represents mean $\pm$ SE (n = 6). ANOVA followed by two tailed Student's t test were used to find out statistical significance at *p<0.05; **p<0.01

cancer (Zhang *et al.*, 2015). Apoptotic tissue lesions, as found in the present histopathological uterine section (Fig. 7), may be validated by the elevated serum LDH level (Gimeno *et al.*, 1979) following arsenic ingestion (Fig. 4). Fractal dimension assessment of histological section was recently used to reveal

the fibrotic status of the tissue and in the present study found an increase in fractal dimension in the stained tissue of arsenic treated group (Fig. 6). This may indicate that there may be the increased fibrosis of the uterus in arsenic treated group as a probable consequence of increased collagen deposition in the

Fig. 3 : Total peroxidase activities in cell free extract [A] protected better with comparatively higher doses of vitamin B₁₂ (0.07 µg) and folic acid (4.0 µg). Electrozymogram showing the total peroxidase profile [B] using tissue extracted protein and these two above higher doses of B vitamins considered in the zymogram. In panel [C] relative densitometric values of the peroxidase band from the experimental group, as % of the control lane are shown. Each bar represents mean of six replicates. ANOVA followed by two tailed Student's-t test were used to find out statistical significance at *p<0.01; **p<0.001

uterus (Zouein *et al.*, 2014). Significantly increased LDH level (58%) in arsenic exposed rats in the present investigation (Fig. 4) may play a decisive role in the fibrotic changes of the organ by stimulating collagen deposition (Judge *et al.*, 2015). Deficiency of these two B vitamins may be accounted for the elevation of serum LDH level (McCarthy *et al.*, 1966). Hence, maintenance of adequate levels of these B vitamins may play an imperative role to prevent uterine necrosis.

Since estrogen induces peroxidase activity in uterine cells; hence, estrogen plays a decisive role in the restoration uterine peroxidase activity as well as uterine development and proliferation (DeSombre and Lyttle, 1979). Another possibility for such uterine cellular malformation (Fig. 6) and reduced

Fig. 4 : Total serum LDH activity illustrated for the extent of cellular damage. [A] is showing enzymatic activity in the cell free assay system following dose dependent co-administration of B vitamins in arsenic intoxicated rats. vitamin B₁₂ (0.07 µg) and folic acid (4.0 µg) had comparatively higher effectiveness in the restoration of arsenic induced tissue damage. Hence, in panel [B] these above two doses of vitamins were considered and serum protein in each lane was electrophoresed on agarose gel followed by substrate specific development of LDH bands. Each bar represents mean mean of six replicates. ANOVA followed by two tailed Student's-t was used to realize the statistical significance at *p<0.05; **p<0.01

organ weight (Table1) in arsenic treated group may be the consequence of downregulation of plasma estradiol signaling (data not shown) due to the generation of free radicals as indicated by the existence of asynchronized estrous cycles (4.1 ± 0.2 cycles per animal); dominated by consistent diestrus, following arsenic exposure when compared with control (7.2 ± 0.2 cycles per animal) in the present study. Data from the previous studies implied that the inhibitory role of sodium arsenite on key ovarian steroidogenic enzyme (Δ^5 , 3 β -HSD and 17 β -HSD) activities may interrupt ovarian estradiol synthesis (Chattopadhyay and Ghosh, 2010; Chattopadhyay *et al.*, 2003). Additionally, low plasma levels of FSH and LH downregulate the activities of these steroidogenic enzymes (Chattopadhyay and Ghosh, 2010; Chattopadhyay *et al.*, 2003).

Fig. 5 : Vitamin B₁₂ and folate effect on the DNA fragmentation and the comet formation in uterine cells of rat treated with arsenic in a dose dependent manner. The uterine horn cells were used for DNA preparation; tissue was treated with lysis buffer (50 mM Tris pH 8.0, 20 mM EDTA, 10 mM NaCl, 1% SDS, and 0.5 mg ml⁻¹ proteinase K) and centrifuged followed by an extraction with phenol-chloroform mixture. The band intensity of DNA at its different positions on the agarose gel is evaluated. Lane distribution: control rat (lane A) or treated with As³⁺-0.4-ppm (lane B) or As³⁺-0.4-ppm+ vitamin B₁₂-0.04 µg (Lane C) or As³⁺-0.4-ppm plus Folic acid-2.0 µg (lane D) or As³⁺-0.4-ppm + vitamin B₁₂-0.04 µg + Folic acid-2.0 µg (lane E) or As³⁺-0.4-ppm+ vitamin B₁₂-0.07 µg (Lane F) or As³⁺-0.4-ppm + Folic acid-4.0 µg (lane G) or As³⁺-0.4-ppm+ vitamin B₁₂-0.07 µg + Folic acid-4.0 µg (lane H)

Uterine injury was primarily due to the remarkable loss of secretory cells of uterine tissue along with the distortion of endometrial layer. B₁₂ and folic acid co-treatment significantly secured such As³⁺ induced uterine-disorders (Fig.6). These defensive mechanisms of B₁₂ and folate might have ended arsenic induced apoptotic stimuli and sheltered uterine tissues from the necrotic damages since there was an improvement in the uterine histopathological status (Fig.6) and DNA smears (Fig.5) in

response to these vitamins' co-administration in As³⁺ ingested rats. Further studies are necessary to make a more conclusive report. Significant drop in the fractal dimension of uterine tissue in a dose dependent manner, following the treatment with vitamin B₁₂ and folate alone or in combination with arsenic exposed rats (Fig. 5-6) was also observed. It may be postulated that possible fibrous change due to probable excess collagen deposition in the uterine tissue may be reversed by the co-administration of vitamin

Fig. 6 : Uterine tissue was implanted in paraffin, serially sectioned laterally at 5.0 μm , stained with eosin and haematoxylin (Harris) and observed under a microscope (magnification 340) to study the uterine histoarchitecture (A-H). Secretory nature of uterine bed was lost following arsenic intoxication (panel-B) and gradual and or significant reversal of secretory tubules was observed following the treatment with these B vitamins (panel E-H). Arrow denotes secretory vesicles. Panel distribution: control rat (panel A) or treated with As^{3+} -0.4-ppm (panel B) or As^{3+} -0.4-ppm+ vitamin B_{12} -0.04 μg (panel C) or As^{3+} -0.4-ppm plus Folic acid-2.0 μg (panel D) or As^{3+} -0.4-ppm+ vitamin B_{12} -0.04 μg + Folic acid-2.0 μg (panel E) or As^{3+} -0.4-ppm+ vitamin B_{12} -0.07 μg (panel F) or As^{3+} -0.4-ppm + Folic acid-4.0 μg (panel G) or As^{3+} -0.4-ppm+ vitamin B_{12} -0.07 μg + Folic acid-4.0 μg (panel H). Graphical representation denotes fractal dimension of uterine bed was increased in As^{3+} ingested rats. Subsequent co-administration of vitamin B_{12} and folic acid gradually and dose dependently decreased the fractal dimension from intoxicated state to convalescent state. NB: There is no unit for Fractal dimension, this is ratio

B₁₂ and folate by reducing the level of circulating homocysteine (Jayarajah, 2005).

However, animals in the vitamin-supplemented group showed a re-established morphology as compared to those in arsenic-exposed group. In our previous investigation, we confirmed 7 estrous cycles with low dose (0.4 ppm) of arsenic treatment significantly increased arsenic concentration in uterine tissues (Chattopadhyay and Ghosh, 2010). A high tissue arsenic concentration is obviously allied with the impaired antioxidant status and tissue damages. This most likely reflects the perception that inorganic arsenic may be associated with reduced plasma level of vitamin B₁₂ and folate, thereby reducing the arsenic detoxification by a delay in the biliary excretion of arsenic (Kile and Ronnenberg, 2008). Hence, exogenous vitamin B₁₂ and folate may promote biliary excretion of arsenic in the methylated form (As III).

Methylation of arsenic has been implicated in the excretion, removal and detoxification of arsenic from the system. Methyl cobalamin (CH₃B₁₂), a derivative of vitamin B₁₂, and reduced glutathione (GSH) are projected as the coenzymes and are required in the methylation of inorganic arsenic in association with SAM (Nakamura, 2011). It has already been explored that arsenic is detoxified through methylation reaction involving S-adenosylmethionine and the enzyme methyl transferase. In addition, SAM contributes methylation of As (III) via one-carbon metabolism to yield methylarsonic acid (MMAs) and S-adenosylhomocysteine followed by the generation of homocysteine and adenosine by the hydrolysis of S-adenosylhomocysteine (Hall and Gamble, 2012). This is managed largely by downstream remethylation of homocysteine by methionine synthase using N₅-methyltetrahydrofolate as a co-substrate which is crucial for the regeneration of SAM and removal of arsenic. Hence, folate metabolism extremely requires the involvement of methionine synthase for the maintenance of folic acid pool in the body. Folic acid also supports the maintenance of endogenous methionine level by the conversion of homocysteine to methionine. Arsenic is necessarily oxidized from its (III) to (V) state during the process of its methylation-driven detoxification. Their intracellular levels are regulated by folate and B₁₂ (Kligerman *et al.*, 2003). This study advocates a possible effective drainage of free radicals, which might be helpful in tissue/ DNA protection.

Removal of arsenic from the organs is important for reducing uterine stress and normalization of its morphology. However, the synthesis of endogenous methionine from S-adenosylhomocysteine is ultimately catalyzed by the expansion of endogenous methionine due to the involvement of B₁₂ as a cofactor for methionine synthase. Hence, the therapeutic measures retrieved by vitamin supplementation at physiological concentration in this study may promote the methylation process and make it possible to eliminate arsenic through urine. As a

consequence of low circulating level of folate increased tissue retention and toxicity of inorganic arsenic is possible due to the restricted biotransformation and excretion of inorganic arsenic. Therefore, arsenic methylation, as well as removal demands the participation of folate and homocysteine in this detoxification process (Spiegelstein *et al.*, 2003).

Nonetheless, the restricted generation of free radicals in arsenic treated group is validated in the present investigation following the co-treatment of these exogenous vitamin B₁₂ and folate. These vitamins are also allied to the defense of the uterine tissues and genetic materials ensuing in the hindrance of necrosis and probable carcinogenesis (Mukherjee *et al.*, 2006).

Furthermore, rebuilding of uterine weight in arsenic affected rats following vitamin B₁₂ and folate co-treatment (Table-1) may be due to the safeguarding of ovarian steroidogenesis and plasma gonadotrophin levels. Previous studies have reported that vitamin B₁₂ and folate may play a crucial role in restoring peroxidase activities in arsenic treated rats (Stokstad *et al.*, 1980), and the results in this regard also strengthen this interaction between these two vitamins and peroxidase. In vitamin B₁₂ and folate co-administered group alone (Vit B₁₂ - 6.0 ± 0.4 cycles/animal; folate- 6.3 ± 0.2 cycles / animal) or in combination, the number of estrous cycles was not disparate (7.0 ± 0.2 cycles / animal) from the control level in response to the probable consequence of the preservation of estradiol signaling (Bennett, 2001; Chattopadhyay and Ghosh, 2010). Cervical dysplasia characterized with the abnormalities of the cell lining of the cervix and uterus is the probable consequence of B₁₂ deficiency. Folate deficiency reduced circulating testosterone, estradiol and LH levels (Lepkovsky *et al.*, 1951; Wallock Montelius *et al.*, 2007).

We conclude that, folate and B₁₂ co-treatment support cellular synthetic mechanism, as well as protect genetic constituent. These two B vitamins execute a role of strong prevention of necrotic tissue degeneration, perhaps by removing arsenic from the uterine tissues. This defense was accommodating the regeneration of tissue structural materials and the amino acid pool. The clinical implications of this study focus on the enormous possibility of combination therapy with important vitamins or other micronutrients or active exogenous ingredients as a nutritional supplement against arsenic-induced organ toxicity and carcinogenesis. However, arsenic is detoxified in the human body during the course of methylation process and methionine plays an imperative role in this. It is suggested that Vitamin B₁₂ and folic acid facilitates detoxification of arsenic, and hence, reduces the arsenic induced oxidative stress in uterine tissue although more extensive study is required in this field. Finally, the outcome of the study will be helpful in developing a non-invasive treatment strategy among arsenic affected population from the angle of curing arsenic mediated reproductive disorders.

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