Research Article

Ameliorative Effect of α-tocopherol and Ascorbic Acid on the Accessory Reproductive Glands of Adult Male Rats in Arsenic Toxicity

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ABSTRACT

The present study was designed to evaluate the ameliorative effect of α-tocopherol and ascorbic acid in combination, on the male accessory reproductive glands like prostate and seminal vesicle of adult Wister rats in arsenic toxicity. Rats were divided into three groups and maintained as control, treated (As$_2$O$_3$, 3mg/kg b. wt./rat/day), and supplemented (in water and (α-tocopherol, 400 mg/kg b. wt./rat/day and ascorbic acid, 200 mg/kg b. Wt./rat/day) and the level of oxidative stress as induced by arsenic toxicity was evaluated by measurement of prostatic superoxide dismutase (SOD), catalase, glutathione-s-transferase (GST) and acid phosphatase (ACP) activities. DNA damage of prostatic cells was measured by comet assay. Results showed that arsenic trioxide treatment for 28 consecutive days caused significant decrease of weight of both prostate and seminal vesicle, which revived almost at normal level after simultaneous supplementation of α-tocopherol and ascorbic acid. Prostatic SOD, catalase, GST and ACP activities which were greatly reduced in arsenic toxicity, were seen to be significantly restored in supplemented group. Comet assay proved that arsenic induced toxicity was at DNA level which was also ameliorated in the co-administered group. So, the co-treatment of α-tocopherol and ascorbic acid can protect the antioxidant system, and be able to restore the functions of prostate and seminal vesicle of adult rats in arsenic toxicity.

Keywords: Arsenic, Ascorbic acid, Oxidative stress, Prostate, Seminal vesicle, α-tocopherol

INTRODUCTION

Arsenic is a toxic heavy metal abundant in earth crust causing environmental pollution by contaminating the air, water and soil. As heavy metals cannot be destroyed or degraded, they are the persistent environmental contaminants. Moreover, arsenic is widely used as food preservatives, herbicides, rodenticides, insecticides[1] and arsenic containing drugs,[2] which directly or indirectly affect the human health. Ingestion of contaminated drinking water is the major route for human exposure to arsenic.[3] Population exposed to arsenic contaminated drinking water includes those in Taiwan, China, Europe, United States, Bangladesh, and India.[4] Unfortunately in India, arsenic toxicity is increasing rapidly, mostly in the different districts of West Bengal, Bihar, Uttar Pradesh and even Assam. The current maximum permissive contaminant level of arsenic in drinking water, as set by the United States Environment Protection Agency (USEPA) and the World Health Organization (WHO) is 10 μg/L.[5,6] The contamination from natural sources in different geographically distant areas of India can reach hundreds of μg/L.[7]

Chronic arsenic exposure may exert serious harmful effects on skin, red blood cells, nervous system and different metabolic organs.[8] The manifestations of dermatitis, hyperkeratosis, gangrene and skin cancer are common in chronic arsenic exposure.[9] Reproductive system is also affected by arsenic intoxication. It has been reported that chronic exposure to arsenic is associated with decrease in testes and accessory sex organ weights,[10] the alteration of normal sperm morphology[11] and the decreased activity of antioxidant defence system in testis.[12]

The toxicity also causes spermatogenic arrest and increased sperm pathologies.[13] Alteration in the levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone and also the massive degeneration of the germ cells in seminiferus tubules are also reported with this arsenic toxicity.[14] Oxidative stress and the generation of reactive oxygen species (ROS) could also be a consequence of arsenic exposure.[15] Arsenic alters many protein functions by binding its thiol groups[16] and can also causes DNA damage by ROS generation thus alters cellular functions.[17]

The integrity of sperm DNA is an important factor for the success of fertilization as well as normal development of the embryo, foetus and child.[18] The normal functions of accessory reproductive glands are also essential for successful fertilization. The literature survey reveals very little information regarding the possibility of functional alterations of these organs under arsenic-affected state.

It is well documented that arsenic induced cellular alterations are mediated through generation of reactive oxygen species (ROS) and α-tocopherol, as a potent antioxidant, protect the organs against oxidative stress via the inhibition of propagation of ROS reactions.[19] It has been reported by us, that the co-supplementation of α-tocopherol (AT) and ascorbic acid (AA) in arsenic toxicity, causes the reduction of testicular oxidative stress and

restore the altered sperm parameters.[20] The effect of only AA alone is less effective than the combined effect of AT-AA as AT works as a major chain terminating antioxidant and inhibit polyunsaturated fatty acid peroxidation.[21]

It is farther reported by us that AT-AA could not only restore the testosterone level in the arsenic treated rats but also decreases the DNA damage in testicular tissue.[21]

Prostate and seminal vesicle are the two major accessory glands of male reproductive system, which are under direct control of testicular androgenic capacity.[22] Till date there are no available reports regarding the amelioration by the combined application of antioxidant vitamins against the toxic effects of arsenic on accessory sex organs. Therefore, the present study has been designed to evaluate the effect and nature of arsenic toxicity on the male accessory sex organs of rat and also to explore whether the detrimental effects (if any) of arsenic can be protected by the co-administration of AT-AA.

MATERIALS AND METHODS

Experimental design

Twenty four adult male albino rats of the Wister strain of 130 ± 10g body weight were taken for this experiment. The animals were maintained under standard laboratory conditions (14 hrs light: 10 hrs dark, 25 ± 2°C) with free access to food and water. All animals were maintained and sacrificed according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC) guided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India. For the experiments, rats were randomly selected into three groups consisting eight rats in each: group I, control; group II, arsenic treated; and group III, arsenic + α-tocopherol and ascorbic acid supplemented. All the rats were provided with a control diet composed of 71% carbohydrate, 18% protein, 7% fat, and 4% salt mixture and vitamins. For chronic oral exposure to arsenic the dose of 3 mg/kg body wt/day/rat was selected as reported earlier.[20,21] Accordingly, rats of group II and III were orally treated with aqueous solution of arsenic trioxide, for 28 days. The rats of group III, in addition, were treated with α-tocopherol (400 mg/kg body wt/day, dissolved in olive oil) and ascorbic acid (200mg/kg body wt/day, dissolved in water) by oral gavage for once a day. To overcome the impact of any altered food intake, control rats (group I) were pair-fed with other experimental groups, II and III. Food and water along with the body weight of the rats were monitored throughout the experimental period. Rats were sacrificed 24 hrs after the last treatment by light ether anaesthesia. The prostate and seminal vesicle were dissected, weighed and were kept at -20°C for different biochemical assays.

Reagents

Arsenic trioxide was used for this experiment was purchased from Loba Chemical Pvt. Ltd., Mumbai, India

Alfa tocopherol was purchased from Himedia, Mumbai, India and L-ascorbic acid were purchased from SRL Pvt. Ltd., Mumbai, India.

Prostatic superoxide dismutase (SOD) activity

Prostatic SOD activity was measured biochemically.[23] The tissue was homogenized in an ice cold medium containing 0.1M phosphate buffer (pH 7.2) at a tissue concentration of 50mg/mL. The homogenate was centrifuged at 10,000 ×g for 20 min at 4°C. In a spectrophotometer cuvette, 3ml of 0.05M phosphate buffer with EDTA was taken and then to it 50µl of haematoxylin was added and the enzyme activity was assessed spectrophotometrically (JASCO v-530 Easton, MD) at 560 nm by measuring auto-oxidation of haematoxylin with or without 100 µl of tissue homogenate.

Prostatic catalase activity

The prostatic catalase activity was measured from all groups estimated biochemically.[24] Prostate was homogenized in ice cold medium containing 0.05 M Tris HCl buffer (pH- 7.0) at a tissue concentration of 20 mg/ml. The mixture was centrifuged at 10,000 ×g for 20 min at 4°C. In a Spectrophotometric cuvette 0.5 ml hydrogen peroxide solution and 2.5 ml double distilled water were mixed well and read at 240 nm (JASCO v-530 Easton, MD). Then 40 µl of tissue homogenate supernatant was added, mixed well and six readings were taken at 30 second intervals.

Prostatic glutathione s-transferase (GST) activity

Glutathione-S-transferase activity in the prostate tissue samples was measured spectrophotometrically[25] using CDNB (1-chloro-2, 4-dinitrobenzene) as a substrate. The formation of the product of CDNB, S- 2, 4 dinitrophenyl glutathione, was monitored by measuring the net increase in absorbance at 340 nm (JASCO v-530 Easton, MD) against the blank. (The enzyme activity was calculated using extinction coefficient $\epsilon = 6.9$ M^{-1} cm^{-1} and expressed as mM of product formed /min/mg of protein). The amount of protein present in the tissue was measured using standard Lowry method.[26]

Prostatic acid phosphatase (ACP) activity

The measurement of the activity of prostatic acid phosphatase was done following the standard biochemical procedure .[27] Prostate was homogenized in 0.02 M Tris HCl buffer, (pH 7.5), at a tissue concentration of 10 mg/ml. Tissue homogenate was added in a centrifuge tube containing 1 ml buffer [p- nitrophenol phosphate (PNPP) in 0.1 M acetate buffer, pH 5.0]. The mixture was incubated at 37°C for 30 min in a water bath. Then the reaction was terminated by addition of 0.1 ml 0.1M NaOH. The assay was based on the formation of p-nitrophenol (PNP) from the hydrolysis of PNPP. The activity was measured using a spectrophotometer (JASCO v-530.Easton, MD) at 420nm. The concentration of the sample was obtained from a standard curve and expressed as µg of PNP liberated/ mg of tissue/ hr.

Seminal Vesicular Fructose Assay

The seminal vesicle was homogenised in 5% perchloric acid (100 mg tissue/ ml perchloric acid) and the protein free extracts were obtained by centrifugation at 3000 rpm for 10 min. and 2 ml of clear supernatant was taken for analysis. With this supernatant resorcinol (0.1%) and 3 ml HCl (30%) were mixed and kept for 10 min at room temperature and then at 80°C for 1 hour. The Spectrophotometric reading was taken at 515 nm with reference to standard fructose following the standard protocol[28] using the same spectrophotometer (JASCO v-530 Easton, MD).

Prostatic single cell gel electrophoresis

Prostatic tissues were minced and suspended (2 g/ml) in cold HBSS (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$) containing in addition 20 mM EDTA and 10% DMSO. Following a standard method[29] the tissues were allowed for 15 minutes to settle down, then 5 - 10 µL of the cell suspension was taken and mixed with 75 µL 1% low melting point agarose (LMPA) and was embedded in a thin 1% normal melting point agarose (NMPA) on a microscope slide. All cellular proteins were then removed from the cells by adding lysis buffer (containing 2.5 M NaCl, 0.1M Na_2EDTA, 10 mM Trizma base, 1% TritonX and 10% DMSO) and refrigerated overnight. DNA was allowed to unwind under alkaline conditions. Electrophoresis was performed for 20 minutes under alkaline condition (300mM NaOH, and 1mM EDTA, pH>13) at 280mA and 24V (~0.74 V/cm). The broken DNA fragments or damaged DNA undergoing electrophoresis migrate away from the nucleus. In this process, the smallest fragments travel the farthest. Following electrophoresis, slides were neutralized using neutralizing buffer (0.4 M Tris, pH 7.5) and stained with ethidium bromide (2 µg/mL). Images were captured with a fluorescent microscope (Leica DM 3000) using Leica Q Win Plus, Leica Microsystems, Switzerland. Head and tail lengths of 50 randomly selected cells were evaluated by 'COMET SCORE' software (TriTek Corp., Sumerduck, VA).

Statistical analysis

The data were expressed as mean ± SEM (Standard error of mean). For statistical analysis, the quantitative data of each parameter from the different groups were analyzed by Student's t-test. The mean ± SEM was calculated for each group and the corresponding level of significance was calculated. Statistical software system Minitab version 2009 was employed in the entire methodologies. $p<0.05$ level has been considered, in this particular study, as significant minimum.

RESULTS

Food consumption and weight variations

There were no differences in food consumption among the groups throughout the experimental schedule. Arsenic treatment caused significant reduction in the wet weight of prostate and seminal vesicle ($p<0.05$ in both cases) in comparison to that of the control. Arsenic-exposed but AT-AA supplemented rats, by contrast, experienced no such decrease in the prostatic and seminal vesicular weight and those were significantly ($p<0.05$ in both cases) higher than that of the arsenic treated group (Table 1).

Table 1: Gravimetric index report of prostate and seminal vesicle of rats co-supplemented with α- tocopherol and ascorbic acid in arsenic induced state.

Treatment	Relative weight (mg/g b. wt.)	
	Prostate	Seminal vesicle
Control (Gr-I)	$0.166^a \pm 0.012$	$0.342^a \pm 0.032$
As_2O_3 treated (Gr-II)	$0.113^b \pm 0.012^*$	$0.226^b \pm 0.032^*$
As_2O_3+AT-AA (Gr-III)	$0.165^a \pm 0.019^*$	$0.328^a \pm 0.023^*$

Each value represents mean ± SEM (n=8) (Student's t-test). In each vertical column the mean with different superscripts ([a & b]) differ significantly. [$^*p<0.05$]

Prostatic SOD activity

Superoxide dismutase (SOD) activity in prostatic tissue was found to be significantly reduced in the arsenic treated group in comparison to that of the control ($P<0.01$) and the same activity was shown to be significantly restored in the supplemented group ($p<0.05$) (Figure 1a).

Prostatic catalase activity

Prostatic catalase activity was found to be significantly reduced ($p<0.05$) due to arsenic treatment which was significantly restored ($p<0.05$) after supplementation with AT-AA in spite of arsenic toxicity (Figure 1b).

Prostatic GST activity

Remarkable decrease of GST activity ($p<0.01$) was observed in arsenic treated rats when compared with that in the normal rats. The supplementation of AT-AA revealed the restoration of normal activity ($p<0.001$) in spite of arsenic intoxication (Figure 1c).

Prostatic ACP activity

Prostatic ACP activity was found to be significantly reduced ($p<0.001$) due to arsenic toxicity in compared to that of control which was significantly restored ($p<0.05$) due to AT-AA supplementation (Figure 2a).

Seminal vesicular fructose content

Fructose content in seminal vesicle was found to be decreased significantly ($p<0.001$) in comparison to that of control animals. The arsenic treated along with AT-AA supplemented rats experienced significant restoration ($p<0.001$) but not up to normal level (Figure 2b).

Prostatic DNA damage

The fluorescent microscopy of prostatic DNA electrophoresis from rats of all groups revealed significant DNA damage ($p<0.01$) due to arsenic effect but that is

seen to be protected (p<0.01) in AT-AA supplemented rats (Figure-3) as assessed by the comet tail length measurement (Figure 4).
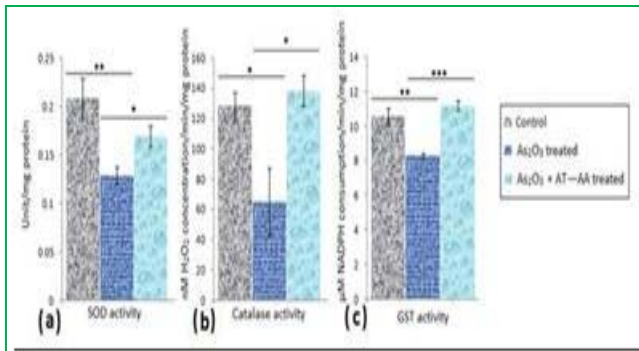

Figure 1: Effect of AT - AA co-administration on SOD activity (a), catalase activity (b) and GST activity (c) of prostate in arsenic affected state of adult male rats. Bars represent mean ± SEM (n=8); Means between the groups were compared with Student's t-test [*p<0.05;** p<0.01;***p<0.001]
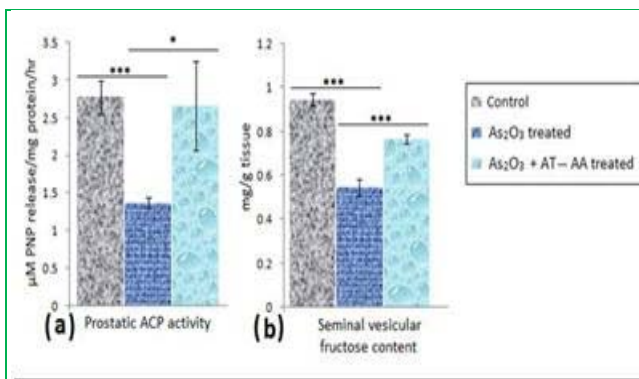

Figure 2: Effect of AT-AA on prostatic ACP activity (a) and seminal vesicular fructose content (b) on arsenic affected rats. Bars represent mean ± SEM (n=8); Means between the groups were compared with Student's t- test [*p<0.05; *** p<0.001]
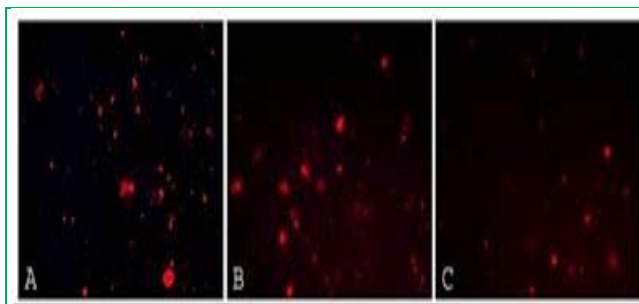

Figure 3: DNA fragmentation in prostate cells by single cell gel electrophoresis in control (A), arsenic-treated (B), and arsenic with vitamin supplemented rats (100X).

DISCUSSION

The present study was focused to evaluate the ameliorative potential of α-tocopherol (AT) and ascorbic acid (AA) against arsenic trioxide induced toxicity of accessory reproductive organs in adult male Wister rats. The results revealed that arsenic toxicity caused

significant decrease of weight of prostate and seminal vesicle and the general body weight remained unaltered which has been mentioned in our earlier report.[21] Supplementation of AT-AA at doses of 400 mg and 200 mg/kg body weight/day respectively for 28 days, prevented the weight decrease of prostate and seminal vesicle in spite of arsenic intoxication.
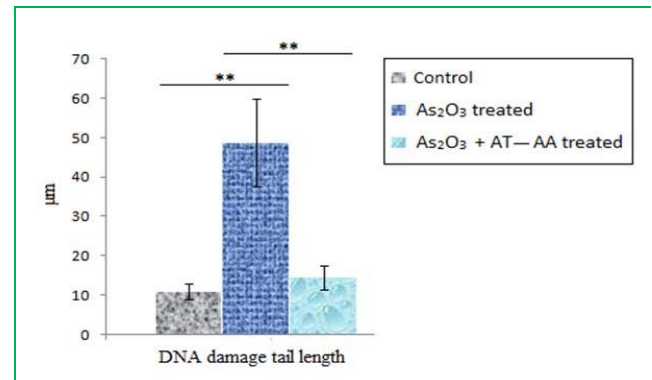

Figure 4: Effect of AT-AA co-administration on prostatic prostatic DNA damage tail length (μm) in arsenic affected rats. Bars represent mean ± SEM (n=8); Means between the groups were compared with Student's t-test [** p<0.01]

Possible protection on arsenic toxicity by the co-administration of AT-AA on testicular histology and sperm parameters was reported from our laboratory.[20] Further protection regarding the spermatogenesis as studied by spermatokinetic analysis, steroidogenesis and the testicular antioxidant enzyme status under similar arsenic insult with same supplementation was also reported by us.[21] Testosterone being the primary androgenic hormone is responsible for the development and maintenance of accessory gland functions and the normal functioning of prostate and seminal vesicle is also well correlated with fertility. Reduced level of serum testosterone due to arsenic effect as reported earlier [21] may be one of the cause behind the decreased accessory glands function as reflected by attenuated prostatic acid phosphatase activity and fructose forming ability of seminal vesicle. Literature survey clearly states that in spite of normal gametogenic activity of testis the reduced secretion of prostate and seminal vesicle hampers normal fertility in men.[30] A large portion of the seminal plasma is contributed by the secretion of prostate and seminal vesicle which is rich in acid phosphatase and fructose, secreted from prostate and seminal vesicle respectively. In our study, reduced activities of prostatic SOD and catalase may be the possible cause behind altered prostate functions in arsenic affected state. Moreover the reduced activity of prostatic GST is in agreement with the ROS induced alteration of prostatic function. On the other hand, fructose in seminal plasma serves to induce the glycolytic metabolism in spermatozoa and in our study due to arsenic insult fructose content of the seminal vesicle has been seen to be decreased. These arsenic induced alterations are in favour of imposing stress on the successful fertility as the available data clearly

indicates that arsenic toxicity causes fertility impairment.[31]

Research data regarding the toxicity of arsenic on accessory glands functioning till date are not available enough. Extensive research on different antioxidants has been started in exploring their efficacy to combat the toxic effects of arsenic on different body systems. The protection from arsenic toxicity by co-administering α-tocopherol with and without ascorbic acid on testicular histology, spermatogenesis and sperm parameters was reported earlier.[20, 32] The present study shows that the co-supplementation of AT-AA can protect the attenuated antioxidant status of prostate and seminal vesicle functions in arsenic intoxicated state.

Arsenic exposure results in oxidative stress due to increased generation increased reactive oxygen species (ROS). Arsenic also alters many protein functions by binding to its thiol groups and can also causes DNA damage by generated ROS, which ultimately lead to altered cellular functions.[33] Studies on testicular tissue from our laboratory clearly states that simultaneous administration of AT-AA can also protect the prostate DNA integrity under arsenic threat. AT- AA administration replenishes the GSH level in arsenic affected state and thus regulates the redox potential of the cell maintaining DNA in native form.[21] The same mechanism may also be effective behind the maintenance of DNA integrity of prostate cells by the AT - AA administration.

Ascorbic acid is the most widely cited form of water soluble antioxidant that prevents oxidative damage to the cell membrane induced by radicals in an aqueous environment [34] and due to hydrophilic nature it is not able to scavenge the radicals within the interior of the membranes where as AT is an efficient scavenger of free radicals within the membranes.[35] Alpha tocopherol having the greatest antioxidant activity among the other vitamin E derivative and also due to acting as a strong inhibitor of polyunsaturated lipid peroxidation[35] is being used synergistically with AA to combat the arsenic induced damages of target organs in different models.[36-37] Here, the protection of accessory sex gland functions is not only due to the known antioxidative capacity but also due to their efficacy in restoring the steroidogenesis in spite of arsenic insult.[21]

Therefore the results of our present study have shown that the combined oral application of α-tocopherol and ascorbic acid can ameliorate the arsenic induced oxidative stress mediated alterations of accessory reproductive gland functions in adult male rats.

CONCLUSION

Our study reveals that arsenic trioxide has detrimental effect on the functions of the male accessory reproductive glands like prostate and seminal vesicle and the co-administration of and α-tocopherol and ascorbic acid, by virtue of antioxidant potential could ameliorate the arsenic effect. Therefore, the clinical trial of this combined therapy may be undertaken on arsenic affected population.

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Source of Support: Nil, **Conflict of Interest:** None.

International Journal of Pharmaceutical Sciences Review and Research
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