Original Article

Immunotoxic and Genotoxic Potential of Arsenic and its Chemical Species in Goats

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ABSTRACT

The study investigated the immunotoxic and genotoxic effect of arsenic and its different species on goats. It was found that arsenic causes haematological crisis. Histopathological changes in spleen and reduced serum immunoglobulin G level without any changes in formazan production in arsenic-treated animals indicated that arsenic is toxic to the humoral immune system. Increased caspase-3 production and higher number of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling)-positive bone marrow cells along with oligonucleosomal DNA fragmentation on agarose gel suggested apoptosis induction by arsenic in the bone marrow cells of goat. Total arsenic concentration in the plasma, bone marrow, and spleen of the exposed group was, respectively, 1.22 ± 0.11, 2.20 ± 0.21, and 3.39 ± 0.14 ppm. Speciation study revealed that arsenite and organoarsenic were the major arsenic species in these samples, suggesting their role in immunotoxic and genotoxic potential in goats.

Key words: Apoptosis, arsenic species, goat, immunotoxicity

INTRODUCTION

Arsenic is considered one of the most toxic elements found in nature that affects more than 200 million people worldwide.[1] The presence of arsenic in ground water is mainly the result of minerals being dissolved naturally from weathered rocks and soils.[2] Humans and animals are mostly exposed to arsenic by consumption of contaminated ground water or through food chain. Once absorbed, arsenic redistributes itself to nearly the entire organ systems of the body and causes toxicity to different organ systems, leading to melanosis, de-pigmentation, and cancer of the skin and various organs, including the liver and lungs, in humans.[3] Abnormalities of the peripheral vasculature, cerebrovascular diseases, and reproductive failure are also seen in people exposed to arsenic.[4,5] It is observed that animal rearing in arsenic prone areas do not show any specific clinical symptoms due to arsenicosis, although toxic outcomes in biological systems have been reported.[6] Arsenic is immunotoxic in nature.[7] It has been observed that arsenic modulates lymphocyte co-receptor expression and release of cytokines in mammals, resulting in immunosuppression and increased susceptibility to infection.[8] Studies of human and mice have revealed that arsenic exerts its toxicity by generating reactive oxygen species, which play a critical role in the mediation of arsenic-induced apoptosis.[9] Arsenic also induces caspase-3 associated with apoptotic cell death.[10]

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Livestock rearing has played an important role in strengthening the economy of many developing countries. Goats are one of the important livestock, which come of use to the community for their meat, milk, and hides. The effect of stress and reduced immunity on the productivity of these animals and moreover on the economy of the country could not be ignored. Reports suggesting role of arsenic species in producing immunosuppression and genotoxicity in livestock are scarcely available. The present study was therefore designed to find out the possible predominant arsenic species that participate in causing immunotoxicity and genotoxicity in goats.

MATERIALS AND METHODS

Chemicals

Caspase-3 colorimetric assay kit and DNA ladder extraction kit were procured from BioVision (Milpitas, CA, USA). The DeadEnd™ Colorimetric TUNEL System was purchased from Promega (Madison, WI, USA). Speciation cartridges were obtained from Metal Soft Centre (Piscataway, NJ, USA). Other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA).

Animals

Clinically healthy black Bengal female adult goats (1-1½ years age) weighing between 12 and 14 kg were purchased from arsenic-free villages (as described by the Public Health Engineering Department, Govt. of West Bengal) and used in this experiment. They were caged individually in custom-made stainless steel metabolic cages. The animals were stall-fed and water was provided *ad libitum*. The composition of feed was 2 parts wheat husk, 1 part crushed maize, 1 part crushed Bengal gram, and 2 parts green grass. The temperature of the animal room was maintained at 26°C and artificial lighting facilities were provided.

Before starting the experiment, the animals were de-wormed once with a mixture of albendazole and rafoxanide (Vetalben®; Indian Immunologicals, Hyderabad, India) at a dose rate of 7.5 mg kg^{-1} body weight. After de-worming, the animals were acclimatized in the experimental environment for 15 days.

Experimental design

Twelve black Bengal goats were divided into two groups out of which group-II was administered with sodium arsenite ($NaAsO_2$) at a dose rate of 2 mg kg^{-1} body weight daily orally for 84 days,[11] whereas group-I was kept as control without arsenic treatment. Blood (5 ml) was collected from both groups using an anticoagulant on day '0' (before starting the experiment) and day 84 for estimation of haematological parameters. Immunoglobulin G (IgG) level in serum and Nitroblue tetrazolium reduction assay in peripheral blood mononuclear cells were also assessed on the same days as above. All the animals were slaughtered on day 85 and spleen and associated lymph nodes were collected for histopathological examinations. Bone marrow aspirates were collected in phosphate buffer solution (0.02 M, pH 7.2) for detection of apoptosis through caspase-3, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling), and DNA laddering assay. Arsenic and its species were also estimated from plasma, bone marrow, and spleen collected after slaughter due to their involvement in immunity and apoptosis. The institution animal ethics committee approved the entire experimental protocol.

Haematological parameters

Total erythrocyte count, total leukocyte count, and differential leukocyte count were determined using a haemocytometer. Haemoglobin was estimated spectrophotometrically by the cyanomethaemoglobin method.[12]

Quantification of IgG

Serum IgG level was quantified by sandwich ELISA in both groups following the method of Heyman *et al*.[13]

Nitroblue tetrazolium reduction assay

Activity of Nitroblue tetrazolium reduction in peripheral blood mononuclear cells was quantitatively measured according to the method of Imaizumi and Breitman.[14]

Caspase assay

Caspase assay was done according to the manufacturer's instructions using the caspase-3/CPP32 colorimetric assay kit (BioVision, USA). Mean absorbance was recorded at 405 nm.

DNA laddering

DNA laddering assay was done using a commercially available apoptotic DNA ladder extraction kit (BioVision, USA) following the manufacturer's instructions.

TUNEL assay

The bone marrow isolated from control and arsenic-exposed animals was washed, re-suspended in phosphate-buffered saline, and added to poly-L-lysine (1:10 dilution in water; Sigma Aldrich)-coated slides, and processed as per manufacturer's instructions (DeadEnd Colorimetric TUNEL System; Promega). The slides were washed in fresh phosphate-buffered saline and endogenous peroxidases were blocked by 0.3% H_2O_2 (Sigma) treatment for 5 min at room temperature. After staining with diaminobenzidine components, the slides were observed under a light microscope (×40). The total number of

TUNEL-positive cells was counted in five random microscopic fields (×40) per specimen to determine the percentage of apoptotic cells.

Estimation of total arsenic

Total arsenic was quantified in plasma, bone marrow, and spleen by the wet ashing procedure in a hot plate using a tri-acid mixture of nitric acid, perchloric, acid and sulphuric acid (10:4:1) following the method of Datta *et al*.[15]

Arsenic speciation

Speciation of arsenic in plasma, bone marrow, and spleen was done as per the method of Patra *et al*.[11] using an arsenic speciation cartridge (Metal Soft Centre, Piscataway, NJ, USA).

Statistical analysis

Statistical analysis was done by unpaired '*t*' test using SPSS software version 17.0. The level of significance was determined at $P < 0.05$.

RESULTS

Haematological parameters

It is evident in the present study that haemoglobin level, total erythrocyte count, and total leukocyte count significantly ($P < 0.05$) decreased in arsenic-exposed animals with respect to the unexposed group [Table 1].

Immunological parameters

Serum IgG level was significantly ($P < 0.05$) reduced in the arsenic-exposed group following 84 days of exposure [Table 1]. It is also evident from Table 1 that there is no significant ($P < 0.05$) difference in formazan production among the groups.

Histopathology

Sections of spleen and associated lymph nodes of arsenic-exposed animals (group-II) showed degeneration of lymphocytes at the centre, with hyperplasia of the same at the periphery of Malpighian corpuscles [Figure 1]. Increased erythrophagocytic activities of reticuloendothelial cells in the red pulp were evident in spleen. Trabeculae showed interceptal thickening, with fibrous tissue replacing the focal necrotized areas. Intravascular haemolysis was also marked. No significant changes were found in the sections of either spleen or associated lymph nodes of the unexposed animals (group-I).

Apoptosis

The results of the present study show that the mean values of caspase-3 activity in the arsenic-exposed group increased significantly ($P < 0.05$) [Figure 2]. Oligonucleosomal DNA fragmentations of approximately 200 bp size were also evidenced in agarose gel electrophoresis [Figure 3] in the experimental group (group-II). A significantly ($P < 0.05$) higher number of TUNEL-positive cells [Figure 2] was observed under microscope in the bone marrow cells of the arsenic-exposed group.

Total arsenic and its species

Total arsenic concentration in plasma, bone marrow, and spleen was, respectively, 1.22 ± 0.11, 2.20 ± 0.21, and 3.39 ± 0.14 ppm. Speciation study revealed that arsenite

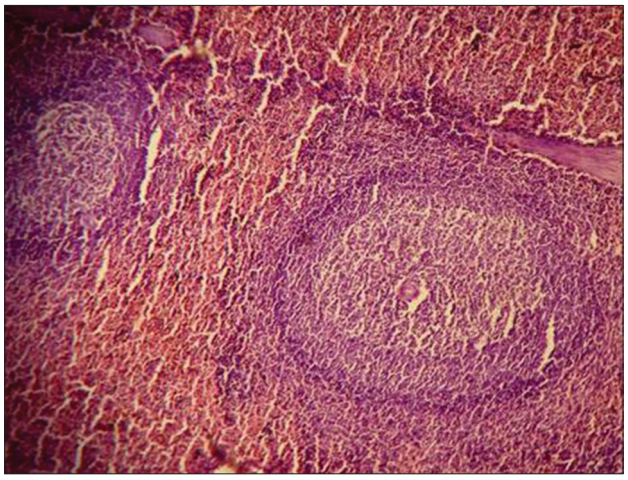

Figure 1: A cross-section of the spleen of the arsenic-exposed group showing lymphocytic degeneration at the centre and hyperplasia of lymphocytes at the periphery of the white pulp (Haematoxylin and Eosin, ×10)

Table 1: Effect of arsenic on different haematological and immunological parameters following administration of sodium arsenite at 2 mg kg^{-1} daily for 84 days in goats

Parameters studied	Day 0		Day 84	
	Group-I	Group-II	Group-I	Group-II
Haemoglobin (g dl^{-1})	8.37±0.15^{x}	8.13±0.15^{x}	8.30±0.21^{x}	5.60±0.40^{y}
Total erythrocyte count (×10^{12} cells l^{-1})	11.28±0.28^{x}	11.18±0.12^{x}	11.15±0.17^{x}	8.20±0.14^{y}
Total leukocyte count (×10^{9} cells l^{-1})	9.31±0.08^{x}	9.42±0.23^{x}	9.32±0.07^{x}	8.48±0.07^{y}
IgG level (mg ml^{-1})	13.08±0.10^{x}	13.15±0.08^{x}	12.94±0.11^{x}	9.10±0.07^{y}
Formazan production (nM ml^{-1})	7.39±0.24^{x}	7.23±0.21^{x}	7.53±0.43^{x}	7.10±0.32^{x}

N.B. Values within a parameter bearing different superscripts (x, y) on a particular day differ significantly ($P<0.05$)

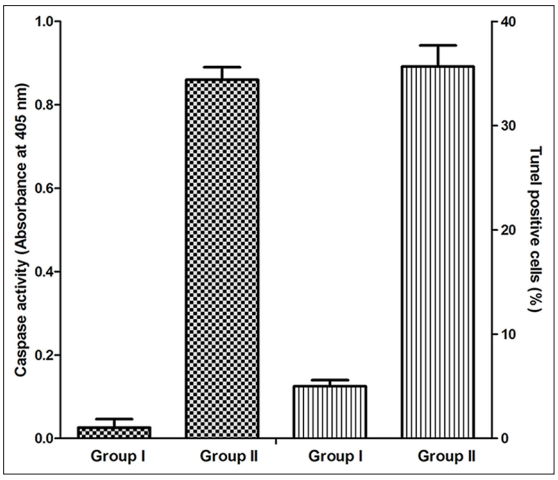

Figure 2: Effect of arsenic on caspase-3 activity and TUNEL-positive bone marrow cells in the unexposed and arsenic-exposed groups

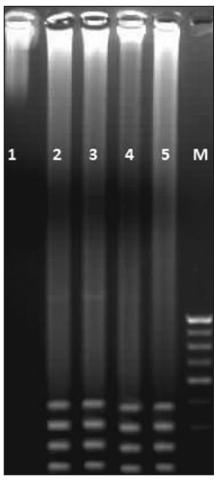

Figure 3: Oligonucleosomal fragmentation and DNA breakage at approximately 200 base pairs in bone marrow cells of the arsenic-exposed group. Lane 1 represents the unexposed group (group-I), whereas lanes 2–5 represent the arsenic-exposed group (group-II). M is a DNA marker (100 bp)

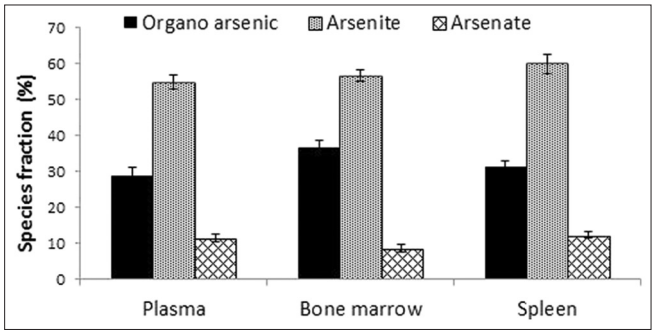

Figure 4: The status of accumulated arsenic species in the arsenic-exposed group following administration of sodium arsenite at 2 mg kg^{-1} daily for 84 days in the goats

and organoarsenic were the major arsenic species that accumulated in the plasma, bone marrow, and spleen. Very less amount of inorganic arsenic accumulated in the above organs [Figure 4].

DISCUSSION

Arsenic is known for its affinity towards the sulphydryl group. In the present study, haemoglobin percentage was much reduced in the arsenic-exposed animals, which might be due to the toxic effect of arsenic on the haem synthesis pathway involving several enzymes that have a sulphydryl group in their moiety. Significant ($P < 0.05$) erythrocytopaenia and leucocytopaenia in the arsenic-treated animals was possibly due to a direct haemolytic or cytotoxic effect of arsenic on the erythrocytes and leucocytes. Several researchers have demonstrated the potential of arsenic in lowering haemoglobin level, and reduction of total erythrocyte count and total leukocyte count.[16-18] Histological aberrations such as lymphocytolysis in spleen and associated lymph nodes as noticed in the arsenic-treated animals were possibly responsible for lymphocytopaenia (as observed in differential leukocyte count, data not shown). Consequently it can be assumed that there may be a sharp reduction of both B and T cells following arsenic exposure. Decreased IgG level – an indicator of humoral response following arsenic exposure – as recorded in the present study supported the assumption. Nain and Smits[19] also reported that arsenic causes suppression of humoral immune response in sub-chronic toxicity in rats. However, no significant change was observed in the cell-mediated immune response as observed in the Nitroblue tetrazolium reduction assay. Interestingly, the spleen section showed increased activity of reticuloendothelial cells in the red pulp, possibly to combat the invading toxicant. Probably this increased phagocytic activity of reticuloendothelial cells restored the cell-mediated immune response which was depleted due to lymphocytolysis in the white pulp. Translocation of cytochrome *c* into cytosol is a primary event that leads to the formation of apoptosomes and activation of caspase cascade. The cascade of cysteine proteases or caspases is a common and critical component of the apoptotic cell death pathway.[20] The higher activity of caspase-3 observed in the present study is a definite indication of ongoing apoptosis. Walker *et al.*,[21] and Yedjou *et al.*,[22] also reported higher caspase-3 activity in the arsenic-exposed cell line. Apoptosis in the bone marrow cells of the exposed group was evidenced by oligonucleosomal DNA fragmentation and TUNEL-positive cells. Arsenic is a potent apoptotic agent,[23] which causes apoptosis by a variety of mechanisms, including production of reactive oxygen species that damage DNA molecules by direct chemical attack.[24] The results of the speciation study revealed that arsenite and organoarsenic are the predominant arsenic species in plasma, bone marrow, and spleen. Therefore, it may be suggested that these two species are mostly involved in immunotoxicity and genotoxicity in goats.

CONCLUSIONS

It may be concluded from the above study that arsenic exposure triggered apoptosis in bone marrow cells,

compromising the humoral immune response, which could possibly be due to arsenic species, arsenite, and organoarsenic species.

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