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Effect of Vitamin E Supplementation on mRNA Expression of Superoxide Dismutase and Interleukin-2 in Arsenic Exposed Goat Leukocytes

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Abstract The aim of this study was to quantify the expression level of genes involved in antioxidant defenses during inorganic arsenic (iAs) exposure in the blood of goats and to evaluate the regulative activity on these genes of antioxidant vitamin E in the diet. Twenty-four crossbred lactating goats (Alpine × Beetal) were distributed randomly into four equal groups (Control, T_1, T_2 and T_3) of six in each, on the basis of average body weight (36.10 ± 0.11 kg) and milk yield (1.61 ± 0.004 kg/day). The animals in T_1, T_2 and T_3 were given 50 mg/kg dry matter arsenic daily, while in T_2 and T_3, vitamin E @100 IU and 150 IU/kg dry matter, respectively, was also supplemented additionally for the period of 12 months. Blood was sampled at 0 day then at 3 months interval and analyzed for the expression level of superoxide dismutase (Cu/Zn SOD) and interleukin-2 (IL-2) using real-time PCR technique. Initially there was no difference ($p > 0.05$) in relative expression of the two genes. But, at 3 months, relative expression of Cu/Zn SOD increased ($p < 0.05$) in T_1 groups then, at 6 and 9 months expression was decreased ($p < 0.05$) in all the iAs treated groups whereas at 12 months, vitamin E supplementation increased ($p < 0.05$) the expression which is comparable to control groups. IL-2 mRNA expression was decreased ($p < 0.05$) at 6 months in all iAs treated groups, at 9 months there was decline trend but not significantly different whereas at 12 months decline trend was less ($p < 0.05$) in vitamin E supplemented groups. The result suggests that vitamin E may have a controlling effect on oxidative stress through modulation of SOD and IL-2 expression.

Keywords Arsenic · Goat · Interleukin-2 · Superoxide dismutase · Vitamin E

Arsenic is a naturally occurring metalloid found in soil, air and water. It is generally prevalent in nature in two forms i.e. inorganic and organic. Organic forms in the environment are generally considered to be non-toxic whereas inorganic forms are toxic. Various studies reported that arsenic could participate in the cellular oxidation-reduction reactions resulting with the formation of excess ROS such as superoxide anion (O_2^-) and hydroxyl radical (OH^-) via a chain reaction (Garcia-Shavez et al. 2006). The rapid elimination of excessive reactive oxygen species (ROS) is essential for organism survival. This is performed by antioxidant system including superoxide dismutase (SOD), catalase (CAT), selenium-dependent glutathione peroxidase (Se-GPx) and metallothionein (MT). SOD are metalloenzymes that catalyses the conversion of superoxide radicals to hydrogen peroxide (H_2O_2) and divalent oxygen (Fridovich 1989). The Cu/Zn-SOD is localized in the cytosol and nucleus, while Mn-SOD is located within the mitochondrial matrix. Subsequently, H_2O_2 is reduced to H_2O by CAT in the peroxisomes, or by GPx in the cytosol. Various stress-related genes were increased in a dose dependent manner in the arsenic-transformed cells, including SOD1 (Cu, Zn-SOD) considered a biomarker for arsenic-induced oxidative stress (NRC 2001; Liu et al. 2001), plays an important role in protecting against oxidant-mediated toxicity associated with arsenic (Lee et al. 2005).

Inorganic arsenic has been established as an immunotoxic agent and affects cellular immune response of innate

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immunity (Sakurai et al. 2006; Lemarie et al. 2006). Arsenic has been reported to be responsible for defective cell mediated immunity and decreased percentage of T helper cells in the body (Yu et al. 2002). IL-2 is produced normally during an immune response and the antigen binding to the T cell receptor stimulates the secretion of IL-2 which in turn enhances the expression of IL-2 receptors (IL-2R). This interaction stimulates the growth, differentiation and survival of antigen selected cytotoxic T cells (Beadling and Smith 2002). IL-2 is also required during T cell development in the thymus (Thornton et al. 2004). Mechanisms by which the metalloid deregulates T cell physiology, and notably IL2 expression, remain however largely unknown. In addition to its general toxicity, chronic exposure to arsenic, impairs lymphocyte, monocyte and macrophage activity in many mammals resulting in immunosuppression (Wu et al. 2003; Duker et al. 2005; Sakurai et al. 2006).

The potential role of oxidative stress in the injury associated with arsenic poisoning suggests that antioxidants may enhance the efficacy of treatment protocols designed to mitigate arsenic induced toxicity. Adequate feeding regimes, nutrient supply and administration of antioxidants, on the other hand, are often claimed as the most appropriate strategies to reduce oxidative stress. Many studies in laboratory animals have underlined the importance of antioxidants in controlling reactive oxygen species (Lu and Foo 2000; Beatty et al. 2000; Bagchi et al. 2000; Agarwal and Rao 2000), but less information is available on the role that these compounds might have in the regulation of oxidative stress in ruminants.

For cattle, sheep and swine maximum tolerable dietary arsenic recommended is 50 mg/kg (inorganic) and 100 mg/kg (organic). The purpose of this study was to evaluate the cumulative effect of recommended inorganic arsenic in a long term exposure. Role of vitamin E which acts as first line of defence in ruminants against pro-oxidant are not much reported. As the vitamin E requirement of all classes goat is 100 IU/kg (Morand-Fehr 1981), therefore 100 and 150 IU/kg doses are decided to see the antioxidant effect. Therefore, the aim of this study was to evaluate the variation, if any, of the level of expression of superoxide dismutase (SOD) and interleukin-2 (IL-2) mRNA in blood leukocytes of arsenic exposed goats and whether antioxidant can reduce that oxidative stress induced by arsenic.

Materials and Methods

Twenty-four crossbred lactating goats (2^{nd} to 3^{rd} stage of lactation) were selected from the Cattle Yard of National Dairy Research Institute, Karnal, India and distributed randomly into four groups (6 each). The distribution of the animals was done on the basis of average milk yield (1.61 ± 0.004 kg/day) and body weight (36.10 ± 0.11 kg). The experimental feeding period was of 12 months. Animals in control (C) group received only basal diet without arsenic supplementation, whereas, animals in group T_1 were given arsenic 50 mg/kg dietary DM as sodium arsenite (s-d-Fine-Chem Limited, India, minimum assay $\geq$98 % purity), group T_2 animals, simultaneously with arsenic exposure, received vitamin E 100 mg/kg DM as dl-α-tocopheryl acetate (Lutavit, BASF, Germany, minimum assay 50 %) and group T_3 animals simultaneously with arsenic exposure, received vitamin E 150 mg/kg DM.

The goats were housed in well ventilated pens with individual feeding. Deworming schedule was followed at the beginning of the experiment and thereafter, at 6 month interval. The experiment was performed with approval from Institute (NDRI, India) Animal Ethics Committee (IAEC No. 28/09-21/11/2009). Before the trial, animals were adapted to the new environment and fed arsenic and vitamin E capsule for 7 days. The animals were fed as per NRC (1981) feeding standard to meet their nutrient requirements. As it was a long term study, two types of fodder either berseem or maize depending upon the season/availability was provided. Concentrate mixture (maize 33 %, groundnut cake (oiled) 21 %, mustard oil cake (oiled) 12 %, wheat bran 20 %, deoiled rice bran 11 %, mineral mixture 2 % and common salt 1 %) having CP 19.81 % and TDN 70 % was procured from Godrej Agrovet Pvt Ltd. Composition of roughage and concentrate mixture was estimated by drawing weekly samples and every effort was made to maintain the supply of the given fodder of similar composition. Content of arsenic in concentrate mixture, berseem and maize were 1.116, 0.10 and 0.09 mg/kg and content of vitamin E were 10.72, 24.86 and 5.99 mg/kg, respectively. The gelatin capsules containing weighed quantity of arsenic and vitamin E were prepared and fed daily to animals in group T_1, T_2 and T_3, respectively.

Blood samplings were done 3 months intervals from the goats. About 10 mL of blood was collected in lithium heparin coated vacutainer tubes (BD Biosciences, USA) from jugular vein of each animal and processed in the laboratory for further analysis immediately after collection.

Total RNA from leukocyte of blood was extracted using Trizol (Invitrogen life science) reagent and the concentrations were measured in Nanodrop (Thermoscientific^{TM}). RNA isolated from blood samples was treated with RNAse free DNAse I (Kappa Biosystem) for removing any genomic DNA contamination. Then first strand cDNA was prepared using random hexamer (Superscript III, Invitrogen life science).

Relative level of mRNA transcripts for target genes was measured by quantitative real-time PCR (qPCR) using the SYBR Green I (Roche) chemistry. The cDNA was used as a template for real time PCR with oligonucleotide primers

designed on the basis of known sequences from other animals. Highly purified salt-free primer for SOD gene (forward primer AGA GGC ATG TTG GAG ACC TG; reverse primer CTG CCC AAG TCA TCT GGT TT), IL-2 gene (forward primer TCG TTG CAA ACG GTG CAC CTA CTT CA; reverse primer TAT GCA TCC TGG AGA GCT TGA GGT TC) and β-actin (forward primer CCA ACC GTG AGA AGA TGA CC; reverse primer CGC TCC GTG AGA ATC TTC AT) were generated and optimized to equal annealing temperature. The amplicon product size was 151 bp for SOD gene, 135 bp for IL-2 and 247 bp for β-actin gene (Fig. 1). For reaction mixture of all the genes, crude first strand cDNA was diluted in 1:4 ratio and used for expression studies. PCR reactions were primed with pairs of gene-specific oligonucleotide primers for target (SOD and IL-2) and housekeeping (β-actin) genes. PCR was initiated by a 'Hold' of 10 min at 95°C followed by 40 cycles of heat denaturation at 95°C for 15 s, annealing at 60°C for 30 s and a 30 s final extension at 72°C. Each sample was run as a technical triplicate of PCR reactions for target gene and a doublet for reference gene. We verified in a preliminary test that our total RNA samples were not contaminated with genomic DNA by running the cDNA synthesis reactions with and without reverse transcriptase. In the later case, there was no product of subsequent PCR reactions with the target and reference gene specific primers. Relative quantification of a target gene to a reference gene was done according to Livak and Schmittgen (2001).

All the experiments were replicated three times. Results were expressed as the mean ± SEM. A difference with value $p < 0.05$ was considered statistically significant. Data were analysed by analysis of variance and statistical differences between the various treatment group means were determined by Duncan's multiple range test (DMRT) using the statistical Product and Service Solutions, Version 17.0.1 software (SPSS Inc., Chicago, IL, USA).

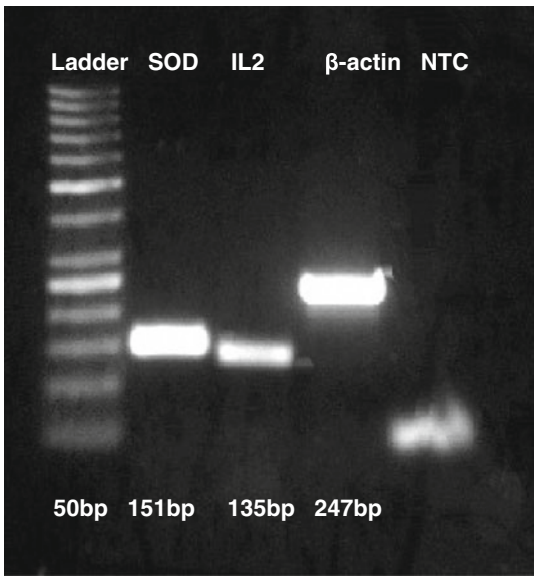

Fig. 1 Agarose gel electrophoresis of Real-time PCR amplified products

Results and Discussion

Relative expression profile of SOD gene has been represented in Fig. 2. Before dietary supplementation, there was no difference in SOD expression between the treatment groups. But, after 3 months of supplementation, compared to control, SOD expression was up regulated/increased ($p < 0.05$) in T_1 groups whereas, in T_2 and T_3 groups expression was statistically similar to control. At 6 and 9 months, T_1, T_2 and T_3 groups expression was decreased ($p < 0.05$) up to the end of 12 months. At 12 months, highest down regulation ($p < 0.05$) was found in T_1 groups whereas in both the vitamin E supplemented groups, expression was similar to control which indicated its antioxidant potentiality to counteract the arsenic induced adverse effect.

In the present study, the up regulation of mRNA expression of SOD gene at 3 months of arsenic feeding might be attributed to increased synthesis of SOD, as a self protective response against oxidative stress (Mates 2000; Pi et al. 2002). This can be confirmed with the erythrocytic SOD activity observed in this study which was elevated after 3 months in arsenic fed groups (data not shown). Similar to our findings, an increase in SOD1 has been reported in humans exposed to arsenic (Lu et al. 2001), and could be an important adaptive mechanism to protect against oxidative damage (Qu et al. 2002). Moreover, arsenic has the potential to activate NF-k beta and the later favors increased synthesis of SOD (Bartoz 1990). 3 Months onwards, down regulation of the gene might be due to over-utilization of antioxidant enzymes resulting failure of adaptive mechanism due to persistent toxicant insult. The above findings are at par with the report of Rana et al. (2010) who reported that supplementation of sodium arsenite @10 ppm in drinking water in rabbit for 12 weeks significantly ($p < 0.05$) down regulated the mRNA expression of SOD2 corresponding to GAPDH control house keeping gene, compared to control lymphocytes. But supplementation of ascorbic acid (25 mg/kg) was able to decrease the oxidative stress and thereby significantly ($p < 0.05$) increased the expression of SOD2 mRNA. Likewise, in the present investigation, vitamin E supplementation increased the expression of SOD due to its antioxidant nature. It is generally believed that the loss of super oxide scavenging activity, which is due to the absence of SOD1 gene (Cu–Zn SOD), leads to a serious burden of oxidative stress (Gralla and Kosman 1992) and the dietary supplementation with natural antioxidants can control oxidative stress through modulation of SOD expression (Colitti et al. 2002). Likewise, Flora et al. (2008) and Park et al. (2009) have demonstrated

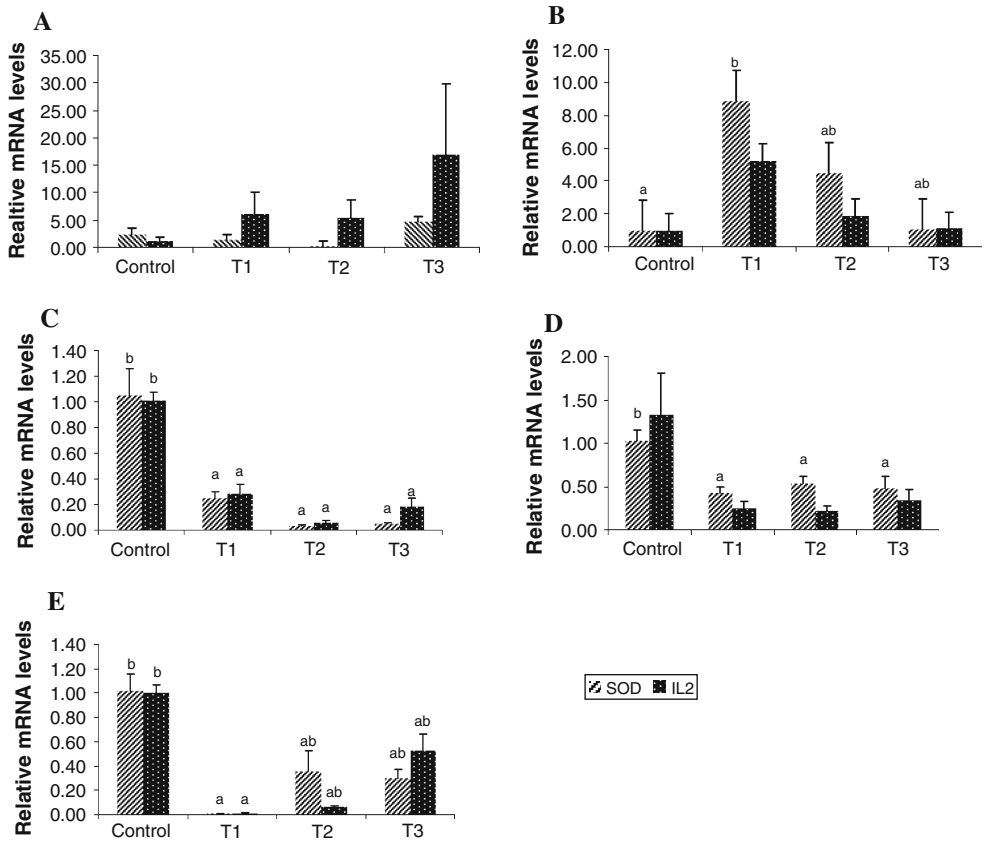

Fig. 2 Relative mRNA expression of superoxide dismutase and interleukin-2, **a** indicates relative mRNA levels at 0 days, **b** indicates relative mRNA levels at 3 months, **c** indicates relative mRNA levels at 6 months, **d** indicates relative mRNA levels at 9 months, **e** indicates relative mRNA levels at 12 months. Data are presented as mean ± SEM. Means with *same letter* were not significantly different ($p > 0.05$)

that antioxidants have the capability to increase the expression of SOD2 gene in arsenic treated cells.

Relative expression profile of IL-2 gene has been presented in Fig. 2. Up to 3 months of experiment, there was no difference ($p > 0.05$) in IL-2 mRNA expression between the treatment groups (T_1, T_2 and T_3). But at 6 months, expression was decreased ($p < 0.05$) in all the dietary treatment groups compared to control. At 9 months expression trend was also less. But at 12 months, vitamin E supplementation in both the doses were able to protect the adverse effect ($p < 0.05$) but between the doses there were no significant difference.

These findings are corroborated with Conde et al. (2007) who reported that sodium arsenite (1 and 10 μM) decreases IL-2 mRNA expression, IL-2 secretion and T cell activation and proliferation in PHA-stimulated murine lymphocytes. Inhibitory effects of arsenite on cell proliferation and IL-2 secretion are a consequence, at least partly of the inhibition of IL-2 mRNA expression and/or alteration of IL-2 mRNA stability. Mechanism of IL-2 mRNA inhibition, the effect could be associated with the capability of arsenite to induce free radical oxygen species (ROS) through interaction with thiol groups. It is well known that endogenous ROS modulate intracellular signals, such as protein tyrosine phosphorylation participating in lymphocyte activation and mitogenesis (Pani et al. 2000; Hardy and Hunt 2004). Besides, arsenic exposure reduces IL-2 production and secretion. The strong inhibitory effects of arsenite on ERK phosphorylation could be related to the inhibition of IL-2 mRNA since previous studies have shown the critical role played by ERK in the transactivation of IL-2 gene and the early T cell activation and proliferation (Crabtree and Clipstone 1994; Modiano et al. 1999). In the present study increased expression of IL-2 gene in the vitamin E supplemented groups might be due to increased lymphocyte proliferation, IL-2 production, and decreased production of the immunosuppressive factor PGE2 (Meydani et al. 1986, 1990). Moreover, vitamin E has been shown to enhance T cell-mediated functions in animals and humans and thereby increases the immunity.

In conclusion present study showed that vitamin E supplementation mitigates arsenic intoxication-induced oxidative damage, which could be due to its antioxidant nature that is having free radical scavenging properties.

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