Studies on Arsenic Toxicity in Male Rat Gonads and its Protection by High Dietary Protein Supplementation

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Abstract: Arsenic was given orally to rats as arsenic tri oxide, 3mg /kg body wt/day in a single dose for 28 consecutive days. This treatment in male Wistar rats caused increase in seminiferous tubular luminal size coupled with reduced accumulation of spermatozoa, and signs of necrotic changes with disarray in cellular organization. Other significant changes were decrease in sperm count, viability and motility ($p<0.001$). On high protein diet (containing pea 37gm/100 gm of diet and casein 9gm/100gm of diet) supplementation along with same arsenic exposure caused partial restoration of normalcy. All these sperm physiological changes and altered gonadal features, both histomorphometric and histological observations, were found significantly ameliorated. Results of this study propose that high protein diet supplementation may be effective to recovery from the toxic effect of arsenic on male gonad of rat.

Introduction

It has become evident that increasing human activities have modified the global cycle of heavy metals and metalloids, including the toxic non-essential elements like arsenic (As) [1]. Among these metals, arsenic exhibits a complex metabolism and is possibly the most abundant and potential carcinogen [2]. Arsenic is present in the nature in stable form as As^{5+} species, and As^{3+}species. An analysis of 25000 tube wells in West Bengal reveals that the average As concentration reaches to 0.3mg/lt of water, and in some places even the concentration reaches up to 3mg/lt of water, where 0.05mg/lt is the permissible limit for drinking water as per WHO [3]. In the process of arsenic metabolism, inorganic arsenic is methylated to monomethyl arsonic acid (MMA) and finally to dimethyl arsinic acid (DMA) followed by a renal excretion. In this process of biomethylation, constant depletion of methyl causes DNA hypomethylation, and thus generates mutation followed by carcinogenesis [2]. Arsenic affects the mitochondrial enzymes, impairs the cellular respiration and causes cellular toxicity. It can also substitute phosphate intermediates, which could theoretically slow down the rate of metabolism and interrupt the production of energy [4]. Male infertility is reflected by low sperm count, low sperm motility and bad quality of sperms (4). Sodium arsenite has been found to have an inhibitory effect on the activity of testicular steroidogenic enzyme Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD) and 17ß-hydroxysteroid dehydrogenase (17ß-HSD) and to reduce the weight of testes and accessory sex glands [4] in rat. High Arsenic level may suppress the sensitivity of gonadotroph cells to GnRH as well as gonadotropin secretion by elevating plasma levels of glucocorticoids. These ultimately lead to the development of gonadal toxicity [4-5]. In recent studies, dietary proteins have been found to have antioxidant activities [6-8]. Wheat (*Puccinia graminis tritici)* and pea (*Pisum sativum)* are both good sources of dietary plant protein, while casein is an animal protein. The antioxidant activities of pea, wheat,

and casein has been studied using different liposomal models and the results show a minimization in lipid peroxidation, thus preventing the damage produced by the free radicals [6]. Our present study has been done to test the causal relationship between arsenic generated oxidative stress and testicular cell damage using rat as a model animal and further to examine whether dietary high protein interventions may be an effective strategy of detoxification that may help in preventing the disorders induced by arsenic.

Materials and Methods:

Animal selection and drug treatment: Normal (Wistar) male rats weighing between 120gm—140gm were selected for our experiment. Animals were maintained in an environmentally controlled animal house (temperature 24 ± 3°C) and in a 12 h light/dark schedule with free access to water. For experiments, rats were randomly selected into three groups consisting six rats in each: group A, control; group B, arsenic-treated; and group C, arsenic + pea + casein-diet supplemented. The animals of groups A and B were provided with a control diet composed of 71% carbohydrate, 18% protein, 7% fat and 4% salt mixture and vitamins [9]**.** For chronic oral exposure to arsenic, a dose was selected (3 mg/kg body wt/day), which is within the range of LD_{50} of a 70-kg body wt human (1–4 mg/kg) and lesser than one-thirteenth of LD_{50} value of rats (40 mg/kg) [6]. Accordingly, animals of groups B, and C were orally treated with aqueous solution of arsenic trioxide, 3 mg/kg body wt/day for 28 days. The animals of group C, in addition, were supplemented with pea (37 g/100 g of diet), which contributed 8.5% protein, and casein (9 g/100 g of diet) which contributed additional 9% protein in the formulation of a high protein (27%) diet [6]. To overcome the impact of any altered food intake, control (group A) animals were pair-fed with other experimental groups B, and C.

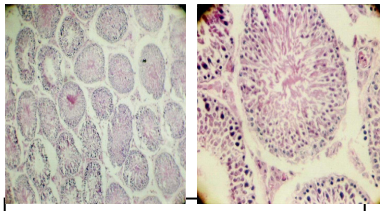
1. *Preparation of permanent slide for histological study of testes:* Testes from all groups of animals were dissected out and were Bouin's-fixed. Paraffin blocks were prepared, and 4–5 μm thin sections were cut with a high precision microtome (IEC Microtome, USA) and routine microscopic slides were prepared. For staining, standard Haematoxylin/Eosin method was followed to study both the histomorphometric and histological alterations.
2. *Sperm viability count:* Immediately after the sacrifice the cauda portion of epididymis was cut. The cauda was kept in 1 ml diluent [10]. This was kept for 5 mins at 37°C. It was then taken out, and an incision was given through the cauda and the sperms were dispersed in the fluid. 40μl of this spermatozoal suspension was transferred to an eppendorf. This was stained with EosinY and Nigrosin (40μl each). This was mixed gently and 25μl from the mixture was taken on a grease free slide and a smear was drawn and dried. The number of viable and non-viable sperms was counted under light microscope.
3. Sperm count: From the dispersed spermatozoal suspension 25μl was charged on a Neubauer Haemocytometer [11] and the numbers of sperms were counted and calculated using WBC chambers.
4. Sperm motility count: The cauda epididymis from all three groups was obtained as mentioned earlier, and each cauda was kept in 1ml PBS 0.2 M, pH 7.4 at 37˚C [12]. 25μl of this was taken on a clean slide covered with a cover slip and was

observed under a microscope. Total numbers of motile sperms per 100 sperms were counted. The readings were taken at the beginning of 1st hour, 2nd hour and 3rd hour of the experiment (the temperature of the spermatozoal suspension was maintained at 37 ^{o} C throughout the process).

5. *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.

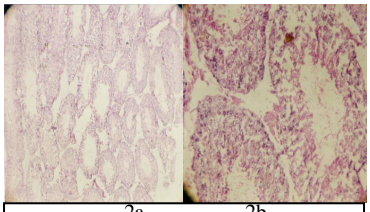
Results:

Histological analysis: The H/E stained histological sections of arsenic treated testes showed increase in luminal areas associated with reduced accumulation of spermatozoa, signs of necrotic changes with disarray in cellular organization (Fig 2) compared to that of control (Fig 1). Protein supplemented rat testes showed the partial recovery compared to the treated group (Fig 3). Recovery includes an accumulation of increased spermatozoa in the luminal areas along with partial amelioration of arsenic induced changes.



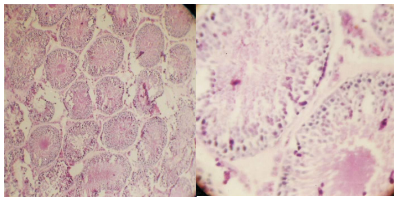
1a 1b

Fig: 1:- Section of control testis showing normal features (a) x10 (b) x40



2a 2b

Fig: 2: - Section of treated testis showing an increase in luminal area, reduced spermatozoal mass, disintegrated cell and nuclear membranes and disorganized cellular orientation.(a) x10 (b) x 40



3a 3b

Fig 3: - Section of protein diet supplemented testis showing features towards normalcy.(a) x10 (b) x 40

Analysis of spermatozoal status :

1. Sperm count: The Table 1 shows that there is a reduction in the number of matured spermatozoa in case of treated group compared to that of the control($p<0.001$). The values from high protein supplemented group, reveals an increased ($p>0.001$) sperm count towards normal. All the values are presented in table1. Results are expressed as mean ±SEM (Standard error of the mean) (n=6).

2. Sperm viability count: The Table1 shows that there is a reduction in the number of viable sperms in cases of treated group ($p<0.001$) and this decrease in viability is minimized ($p>0.001$) in the high protein diet supplemented group. Results are expressed in table 1 following the same statistical analysis as mentioned earlier.

3. Sperm motility count: In case of treated group the decrease in motility was observed in studies done in the 1st, 2nd and 3rd hours compared to control. This decrease was around 10% as observed in three consecutive hours; administration of protein diet minimized the decrease in motility as reflected in the values nearer to that of control. The mean ± SEM (Standard error of mean) was calculated for each

group and the corresponding level of significance was calculated by using the previous statistical analysis.

Table: - 1. Table presenting the effect of protein diet supplementation on the arsenic trioxide induced changes in spermatozoal status in rat: -

Parameters Studied	Control groupA	Treated groupB	Percent decrease %	Significan Level between A&B	Protein diet supplemented groupC	Percentage restored %	Significance Level between B&C
	Mean±SEM	Mean±SEM			Mean±SEM		
1) Sperm count (10^6/cauda)	135.14±5.87	122.24± 5.7	9.5	P<0.001	131.92± 5.31	75	P>0.001
2) Sperm viability (%)	27.16±3.42	14.7± 1.54	46	P<0.001	22.63 ± 3.93	64	P>0.001
3) Sperm motility (%)							
1st hr	97.66 ±0.71	89.16 ± 2.8	8.7	P<0.001	94 ± 1.11	57	P>0.001
2nd hr	81 ±1.01	73 ± 3.2	98	P<0.001	74.5 ± 0.71	19	P>0.001
3rd hr	61.33 ±2.7	54.66 ± 4.5	10.3	P<0.001	54.8 ± 2.98	2	P<0.20

Discussion

Increase in the luminal areas of the seminiferous tubules associated with decreased spermatozoal mass might be due to low levels of gonadotropins in arsenic treated rats, and these low levels are responsible for the decreased production of steroidogenic enzymes [4]. It has been established that arsenic administration leads to decrease in ovarian steroidogenic enzymes synthesis [4]. Thus the low levels of gonadotropins and possibly testosterone might be responsible for the decrease in the spermatozoal mass in the lumen. Decrease in epididymal spermatozoal number provides support towards this histological observation. Arsenic causes lipid peroxidation by generation of reactive oxygen species (ROS) [13-14]. This peroxidation may cause rupture of cell as well as nuclear membrane. This might be responsible for the observed necrosis and disarray in cellular organization in histological section (40 X) Fig-2b. Evidence suggests that arsenic induces free radical formation and thus the generated reactive oxygen species (ROS) react with the polyunsaturatedfattyacid (PUFA) rich spermatozoa, specially the mid spermatozoa and results in peroxidation which finally leads to destruction in spermatozoa causing reduced motility and viability [4]. Pea plus casein diet supplementation along with arsenic treatment reveals that the decrease in sperm count, motility and viability due to toxic effects of arsenic is minimized. As a possible mechanism it could be stated that either pea or casein or both have a recovery role on arsenic tri oxide mediated toxicity by inducing an antioxidant effect against the oxidative stress [15]. The antioxidant properties of milk casein have been established [15]. Studies also indicate that casein phosphopeptides (CPP) and casein hydrolysate bind the peroxidant and thus lipid peroxidation is suppressed [15-17]. Studies for finding the antioxidant mechanisms of pea and wheat have also been done [5]. The pea or pea and casein supplement is effective in reducing the

production of nitric oxide and MDA which could markedly increase the activity of the antioxidant enzymes. This could not only overcome the oxidative stress caused by arsenic but also suppresses the ROS generation from other sources. [6]. Studies on arsenic trioxide induced toxicity on male gonad reveal a good deal of changes in histology of seminiferous tubule, associated with decreased spermatozoal mass. Sperm count, viability, and motility are seen to be affected and the possible mechanisms behind these changes have been discussed. Supplementation of specific proteins with the normal diet causes significant recovery from all these toxic effects. In summary we have demonstrated that the pea and casein by virtue of having antioxidant properties are responsible for restoration of normal gonadal status when supplemented with simultaneous arsenic exposure.

Reference

1. Clarkson, T; Envioron. Health Prospect, 1995, 103, 9—12.
2. Roy, P; Saha, A Metabolism and toxicity of arsenic: A human carcinogen Current Sc. Vol 82 No.1, Jan 2002, Pg-38—45.
3. Mandal,BK, ;Roychowdhury,T, Samanta,G , BasuGK, ChowdhuryPP, Chanda,CR (1996) Current science 70, 976—986
4. Sarkar M, Ray Chaudhuri G, Chattopadhyay A, Biswas NM; Effect of sodium arsenite on spermatogenesis, plasma gonadotrophins and testosterone in rats. Indian Asian J Androl 2003 Mar; 5: 27-31
5. Kreiger DT, Porlow MJ, Gibson MJ, Davis TF , Brain grafts reverse hypogonadism of gonadotropin releasing hormone deficiency. Nature (1982) 298,468-471
6. Mukherjee,S, Das,D Darbar D and Mitra*,C Dietary intervention affects arsenic-generated nitric oxide and reactive oxygen intermediate toxicity in islet cells of rats Current Science, vol. 85, no. 6, 25 September 2003 786-793.
7. S.Maiti and A. K. Chatterjee; Effects on levels of glutathione and some related enzymes in tissues after an acute arsenic exposure in rats and their relationship to dietary protein deficiency. Arch Toxicol 2001 Nov;75(9):531-7
8. P. Y. Y. Wong and D. D. Kitts* Chemistry of Buttermilk Solid Antioxidant Activity J. Dairy Sci. 2003, 86:1541-1547.
9. Chatterjee, A. K., Jamdar, S. C. and Ghosh, B. B., Effect of riboflavin deficiency on incorporation *in vivo* of [14C] amino acid into liver proteins of rats. *Br. J. Nutr*., 1970, 24, 635–640.
10. Jequier A.M., Male Infertility. A guide for clinician. Blackwell Pg: 64—65.
11. Jequier A.M. Male Infertility. A guide for clinician. Blackwell Pg: 55—61.
12. Jequier A.M. Male Infertility. A guide for clinician. Blackwell Pg: 61—63.
13. Xu Y, Wang Y, Zheng Q, Li X, Li B, Jin Y, Sun X, Sun G. Association of oxidative stress with arsenic methylation in chronic arsenic-exposed children and adults. Toxicol Appl Pharmacol. 2008 Jul 1. (Epub ahead of print).
14. Osbaldo Ramos, Leticia Carrizales, Leticia Yáñez, Jesús Mejía, Lilia Batres, deogracias Ortíz, and Fernando Díaz-Barriga : Arsenic Increased Lipid Peroxidation in Rat Tissues by a Mechanism Independent of Glutathione Levels. Environmental Health Perspectives 103, Supplement 1, February 1995.
15. Cervato G, Cazzola R, Cestaro B. Int. Studies on the antioxidant activity of milk caseins. J Food Sci Nutr. 1999 Jul; 50(4):291-6.
16. Taylor MJ, Richardson T. Antioxidant activity of skim milk: effect of sonication**.** J Dairy Sci. 1980 Nov; 63(11):1938-42.
17. Diaz M, Decker EA.: Antioxidant mechanisms of caseinophosphopeptides and casein hydrolysates and their application in ground beef. J Agric Food Chem. 2004 Dec 29; 52(26):8208-13.

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