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Research Paper

# Efficacy of Riboflavin in Combating Arsenic Intoxication In Testes of Mice.

Medical Science

**KEYWORDS :** arsenic, antioxidant, riboflavin, testis, oxidative stress, cytotoxicity

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## ABSTRACT

*Heavy metal toxicity is alarmingly increasing in present day earth. Arsenic is one such heavy metal that is prevalent in Indian subcontinent and is causing serious threat to mankind. Arsenic is a potent gonotoxic heavy metal that causes severe oxidative stress in male gonadal tissue. Total protein content, an indication of metabolic health of an organ, is found to decrease gradually over long term exposure to arsenic. Activities of stress enzymes like acid & alkaline phosphatase and lipid peroxidase are increased significantly in arsenic treated mice, indicating cytotoxic effects of arsenic on testis. Riboflavin is able to ameliorate all such cytotoxic effects towards normal and thus can become an alternative in combating arsenic intoxication in male gonads.*

**INTRODUCTION**: Heavy metals like mercury, arsenic, lead, cadmium, copper etc are non-degradable and most dangerous pollutants on present day earth (Clarkson, 1995). These metals can interfere with the ligands of proteins, especially with the enzymes, cause oxidative stress and interact with nuclear proteins and DNA causing oxidative deterioration of biological macromolecules (Leonard et al., 2004). Arsenic, among the heavy metals, has drawn attention of many Asian scientists because of its high occurrence in this region. Most affected countries include Bangladesh, India, Nepal, China, Afghanistan, Pakistan, Cambodia, Taiwan, Vietnam, Mexico, Chile etc.

Arsenic exists in several chemical forms. The solubility, stability and cellular toxicity of various forms of arsenic are widely different. Levels of human exposure to arsenic can be well understood by studying the chemical forms of arsenic especially the two most available inorganic arsenic species, arsenate ($As^{+5}$) and arsenite ($As^{+3}$), their transformation, persistence and bioavailability (Duker et al., 2004). Arsenic executes both acute and chronic toxicities in almost all vital organs viz, intestine, kidney, skin, liver, heart, lung, nerves, gonads etc. Several remedial measures like chelation therapy, British antilewisite (BAL), calcium disodium EDTA, DMSA, vitamin E & C, b-carotene, melatonin, taurine etc have been attempted in enlivening arsenic intoxication.

Arsenic is a reportedly potent teratogen and a reproductive toxicant. Thus, our present study is aimed at investing the effect of single sublethal dose of $As_2O_3$ on testis and to find out the effect of riboflavin as a protective or repairing agent on the acute cytotoxicity evoked by arsenic-induced oxidative stress.

**MATERIALS AND METHODS**: Inbred strain of male Swiss albino mice, *Mus musculus*, weighing 20±5 g were injected intramuscularly with $As_2O_3$ @2 mg/Kg body weight. Earlier works of Kundu et al, (2000) and LC50 estimates were used as reference to determine the working dose of toxicant. Three fixation intervals, 2 weeks, 4 weeks and 6 weeks, after single dose, were used to study the effect of $As_2O_3$. A batch of $As_2O_3$-injected mice was orally administered with riboflavin @ 0.2 mg/Kg body weight. The oral administration of riboflavin was initiated after few hours of $As_2O_3$ injection and was continued on daily basis for the specific fixation intervals. In simulation with the $As_2O_3$-injected batch and $As_2O_3$-riboflavin-treated batch, a group of control mice were injected with proportional amount of normal saline and were orally administered with water. Mice of all three batches were sacrificed by cervical dislocation at fixation intervals of 2, 4 and 6 weeks.

Body weight and organ weight of all experimental animals were determined gravimetrically with the help of sensitive weighing balance to prepare gonadosomatic indices.

Total quantity of protein was estimated following the method of Lowry et al, 1951. Arsenic accumulation in testis was measured by analyzing acid digested tissue in Atomic Absorption Spectrophotometer (Varian AA 240, Germany) (Pizarro et al, 2003).

Acid phosphatise and alkaline phosphatise activities were determined by the method of Walter & Schutt (1974). Lipid peroxidation was determined by the method of Okhawa (1979).

All data are presented as mean ± S.E. of 10 similar experimental results. Two tailed t-test were conducted to test the significance at $p<0.001$, $p<0.01$ and $p<0.05$ level, between the data of control and that of both treated series (arsenic treated and As+ riboflavin fed groups).

**RESULTS**: The gonadosomatic index, while compared among three experimental groups, express a satisfactory recovery of loss of organ weights in riboflavin fed group to somehow similar levels as the control groups do.

A high rate of arsenic deposition was observed in testis tissues of arsenic treated mice. The accumulation of arsenic was observed to be reasonably high, and total arsenic content in the tissue remained almost unchanged at the prolonged exposure. Riboflavin in observed to cause significant reversal of the condition, but still to reach the normal level even in $6^{th}$ week of treatment.

A gradual and steady fall in total protein content in testis tissue has been observed in $As_2O_3$ injected mice groups as compared to that of saline treated control group. Worst condition arises towards $6^{th}$ week of arsenic exposure. This dramatic loss in total protein content may be correlated with the anomalies related to the testicular proteins and is thought to be due to arsenic intoxication. Riboflavin is observed to cause a steady recovery of total protein content of testis. An interesting finding was recorded in case of $6^{th}$ week of experiment where the protein content of riboflavin-fed group was observed to exceed that of saline-treated control group. This may suggest some influence of riboflavin itself on the maintenance of testicular protein level.

The activity of acid phosphatase in testicular tissues was found to rise sharply even in $2^{nd}$ week of injection with $As_2O_3$ and it rose to a very high level on $6^{th}$ week of injection. The rise in lev-

els of acid phosphatase was calculated to be significant statistically in all exposure schedules. Riboflavin-fed $As_2O_3$treated mice showed a significant recovery of this condition. The acid phosphatase activity in this gonadal tissue dropped down to a level closer to saline treated controls. Strikingly, 2nd week of exposure showed best recovery, by riboflavin, in acid phosphatase level but 4th week showed the worse. This peculiar response of testis tissue might have some relevance with the remedial effect of riboflavin on genotoxicity caused by arsenic.

Alkaline phosphatase activity of testis tissues of mice injected with $As_2O_3$ showed a tendency of gradual rise while compared to the same of control mice in respective exposure intervals. The enzymatic activity of alkaline phosphatase in arsenic affected testis was observed to be high, as much as nine times, that that of control testis at 6th week of experiment. Such high phosphatase activities in successive intervals may be attributed to rise in oxidative stress due to arsenic poisoning in long term exposures. Results from testis tissues of $As_2O_3$ treated mice fed with riboflavin showed a significant recovery along all intervals. 2nd week of treatment with riboflavin gives closer result of alkaline phosphatase activity to that of saline treated control group. 4th week and 6th week of riboflavin treatment also were observed to be significant in reverting the increased alkaline phosphatase activity caused by arsenic intoxication.

Concentration of MDA, an indication of lipid peroxidation, was noted to be higher along all exposure intervals, in the testis tissue of mice injected with $As_2O_3$, as compared to that of saline treated controls. This statistically significant increase was observed in all experimental intervals and in almost equality. Mice treated with arsenic and fed with riboflavin showed a successful reversal of the cytotoxic effects of arsenic on male gonadal tissue. The levels of lipid peroxidation were estimated to be decreased significantly to come closer to that of control groups of respective intervals.

**DISCUSSION**: Arsenic is concerned with very serious health problems. Most of the vital organs are affected by this heavy metal. Toxic effects of arsenic on male gonads are comparatively less documented as compared to that of the other organs like liver or skin. No detailed study has earlier been made on the efficacy of riboflavin to normalise the loss of body & organ weight and the enzymatic malfunctions caused by arsenic intoxication. Arsenic had earlier been reported to cause oxidative stress in several vital organs. Present study is aimed at the extent of remedial efficacy of a potent antioxidant, the riboflavin, against arsenic intoxication in male gonadal tissue.

The alteration in gonadosomatic index indicates that arsenic has caused decreased metabolic activity. General tendency of gain in body weight in control mice was observed to be reverted by the effect of arsenic. This loss of body and organ weight is successive as the experiment progresses. The rapid degeneration of the testicular macromolecules is continued towards 6th week of exposure. Thus it can be said that the single sublethal dose of arsenic has created a long term degenerative effect on testis. Works of Sarkar et al. (2008) suggests cellular regression of testicular tissue, and corroborates to present findings of loss of organ weight as well as body weight.

The heavy degradation of total protein content in $As_2O_3$ affected testis tissue is thought to be associated with the cytotoxic effect of arsenic. Gradual loss of total protein content strongly indicates the prolonged effect of single sublethal dose of $As_2O_3$. The toxic effect of $As_2O_3$ on protein synthesizing machinery can be considered as the reason behind the degradation of testicular protein level.

Very significant and gradual increase in the stress enzymes like acid & alkaline phosphatase and lipid peroxidase denotes cytotoxicity caused due to oxidative stress. Arsenic induces oxidative stress in cells of testis. Prolonged stress of single sublethal dose of arsenic causes the gradual rise in acid and alkaline phosphatase activities towards longer exposures. Lipid peroxidation in testicular cells also expresses a gradually increasing oxidative stress at prolonged exposures. The dramatic rise in the activities of stress enzymes, along the course of exposures, may be attributed to the combination of different signalling malfunctions caused by arsenic and interference of arsenicals to so many proteins that are essential to maintain redox balance in cellular systems.

Results of arsenic accumulation in testis tissue indicate less removal of arsenic from the organ in course of time. Liver and kidney tissues can eliminate heavy metals by scavenging or biomethylation or simple removal. But testis, being a soft tissue, cannot perform such eliminations. Maximum accumulation of arsenic in testis during 4th week of exposure may suggest a probable retention time for arsenic in this specific study schedule.

Riboflavin was selected in present study because of being a potent antioxidant and having been active part of FMN & FAD, enzymes related to reduce oxidative stress. The mice groups treated with arsenic and fed with riboflavin show a gradual and steady recovery of body weight as well as organ weight. The initial loss in total protein content of the testicular tissue also is recovered by riboflavin uptake. The protein synthesizing machinery is thought to be on the track again by the action of riboflavin. The remarkable changes in the activity of stress enzymes in the riboflavin fed group also support that this vitamin has significant ability to regulate the important enzyme systems associated with combating arsenic poisoning. The isoalloxazine ring riboflavin molecule provides it with significantly high affinity to react with molecular oxygens, liberated due to oxidative stress caused by arsenic intoxication. Along with the scavenging function, the flavoenzymes are known to take part in various cellular signalling pathways and maintenance of redox potential in cell. Riboflavin is observed to have the efficiency of lowering the stress caused by arsenite on male gonads even in the 2nd week of exposure. The levels of peroxidases and phosphatases in riboflavin fed arsenic intoxicated testis reach almost near the control values in early intervals of present experiment. Riboflavin also has acted effectively in reducing the accumulation of arsenic in testis. Though, it was not found able to remove all arsenic deposited in testicular tissue, but the rate of removal of arsenic from the said tissue is significantly increased, even in 2nd week of treatment.

**CONCLUSION**: Arsenic has a serious impact on mammalian testis. Present work was attempted to assess the toxicity induced in male gonadal tissue. Arsenic has a prolonged effect on this tissue and the effects are mostly concerned with the oxidative injuries. Heavy metals have a specific retention time in a cell, after which detoxification by methylation occurs to eliminate them. Riboflavin can effectively lower the retention time by scavenging the ROS produced due to arsenic intoxication. The changes in enzymatic activity as caused by arsenic are also effectively normalised by riboflavin. Our study indicates that riboflavin can prevent cytotoxicity and reduce oxidative stress.

Table 1: **Gonadosomatic index** of mice at different fixation intervals

| | 2nd week ± S.E. | 4th week ± S.E. | 6th week ± S.E. |
|---|---|---|---|
| Control | 0.502 ± 0.0052 | 0.499 ± 0.0051 | 0.498 ± 0.0048 |
| Arsenic | 0.488 ± 0.0052 p<0.01 | 0.473 ± 0.005 p<0.01 | 0.468 ± 0.00494 p<0.01 |
| Arsenic + Riboflavin | 0.492 ± 0.00372 p<0.05 | 0.495 ± 0.00462 Non significant | 0.497 ± 0.00437 Non significant |

Table 2: **Arsenic accumulation** (mg/gm tissue) in testis of mice

| | 2nd week ± S.E. | 4th week ± S.E. | 6th week ± S.E. |
|---|---|---|---|
| Control | 0.03 ± 0 | 0.02 ± 0 | 0.02 ± 0 |
| Arsenic | 14.518 ± 0.6124 p<0.001 | 32.1 ± 0.771 p<0.001 | 36.702 ± 1.124 p<0.001 |
| Arsenic + Riboflavin | 10.244 ± 0.622 # p<0.001 | 14.083 ± 0.825 # p<0.001 | 7.044 ± 0.617 # p<0.001 |

# p<0.001 while compared to Arsenic group

**Table 3: Comparison of different biochemical parameters**

| **Total protein content** (mg/gm) in testis of mice at different fixation intervals | | | |
|---|---|---|---|
| | 2nd week ± S.E. | 4th week ± S.E. | 6th week ± S.E. |
| Control | 126.116 ± 1.743 | 135.244 ± 1.524 | 132.59 ± 1.283 |
| Arsenic | 62.612 ± 0.586 p<0.001 | 54.195 ± 0.542 p<0.001 | 38.217 ± 0.439 p<0.001 |
| Arsenic + Riboflavin | 108.078 ± 1.624 p<0.01 | 127.265 ± 1.342 p<0.01 | 139.151 ± 2.041 Non significant |
| Activity of **acid phosphatase** (mM of phenol/100 mg protein) in testis of mice | | | |
| | 2nd week ± S.E. | 4th week ± S.E. | 6th week ± S.E. |
| Control | 0.341 ± 0.0033 | 0.317 ± 0.00316 | 0.311 ± 0.00297 |
| Arsenic | 0.984 ± 0.098 p<0.001 | 2.029 ± 0.02 p<0.001 | 8.277 ± 0.0611 p<0.001 |
| Arsenic + Riboflavin | 0.482 ± 0.0047 p<0.01 | 0.752 ± 0.00691 p<0.01 | 0.589 ± 0.0061 p<0.01 |
| Activity of **alkaline phosphatase** (mM of phenol/100 mg protein) in testis of mice | | | |
| | 2nd week ± S.E. | 4th week ± S.E. | 6th week ± S.E. |
| Control | 0.988 ± 0.0091 | 0.932 ± 0.00924 | 0.898 ± 0.00883 |
| Arsenic | 2.616 ± 0.026 p<0.001 | 3.198 ± 0.0301 p<0.001 | 7.812 ± 0.0778 p<0.001 |
| Arsenic + Riboflavin | 1.081 ± 0.011 p<0.01 | 1.414 ± 0.0121 p<0.01 | 1.486 ± 0.0124 p<0.01 |
| **Lipid peroxidation** (n mole MDA/gm) in testis of mice | | | |
| | 2nd week ± S.E. | 4th week ± S.E. | 6th week ± S.E. |
| Control | 346.152 ± 3.325 | 358.148 ± 3.551 | 357.605 ± 3.483 |
| Arsenic | 504.456 ± 5.102 p<0.001 | 519.135 ± 5.028 p<0.001 | 524.043 ± 5.219 p<0.001 |
| Arsenic + Riboflavin | 361.027 ± 3.523 p<0.01 | 371.253 ± 3.661 p<0.01 | 366.429 ± 3.617 Non significant |

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