Comparative effectiveness of Tiron (4,5-Dihydroxy benzene 1,3-disulphonic acid disodium salt) and CaNa$_2$EDTA with time after beryllium poisoning

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The efficacy of two chelating agents (Tiron and calcium disodium EDTA) in the treatment of beryllium induced blood biochemistry and hepatic histopathological alteration was investigated at different duration in female albino rats. Single administration of beryllium nitrate at a dose of 50 mg/kg (im) showed significant decrease in haemoglobin percentage, blood sugar level, protein contents and activity of alkaline phosphatase. On the contrary significant elevation was found in the activity of transaminases (AST and ALT). Tiron was found to be more effective than CaNa$_2$EDTA in reducing the beryllium induced haematological alterations and histopathological lesions in liver. These findings were further confirmed by AAS thus, in which reduced beryllium body burden was seen in liver and blood with Tiron.

Beryllium is the lightest of all solids and chemically stable substance and is extensively used in missile, nuclear reactor components, aerospace, telecommunications and computer industries and ceramics. Owing to its valuable properties in defense equipment's beryllium assumed an important position in national defence program of India, therefore India has to develop its own beryllium plants. Occupational exposure to beryllium has been associated with various important health syndroms. It causes chronic beryllium disease (CBD) which is a fatal disease of 20th century. Its intoxication causes acute chemical pneumonitis, chronic pneumonitis with granuloma, pulmonary tumors, bone sarcoma and reekets. It has been reported that cell death is due to lowering of blood sugars and liver damage. The principal source of air borne beryllium and its exposure to general population is the combustion of fossil fuels. Tobacco smoking is probably a major source of exposure to general population, 0.074 mg beryllium has been reported to be present in the smoke from one cigarette. Workers are directly exposed in industries which include mining and processing of beryllium alloys. In order to overcome its toxic effects number of chelating agents have been tried but none have met with success. Authors have reported the efficacy of Tiron and CaNa$_2$EDTA against beryllium intoxication in preliminary study, so in the present investigation a comparative detailed study was planned (Fig. 1).

Materials and Methods

Beryllium nitrate was purchased from Fluka (Switzerland). CaNa$_2$EDTA and Tiron were obtained from Sigma Chemicals Co. (St. Louis, USA). Female albino Sprague Dawley rats weighing 120±10 g were selected from our departmental colony and kept under uniform conditions of light (14hr) and dark (10hr) and temperature (22°±2.0°C). The rats were kept on a standard pelleted diet (Lipton's India Ltd., Calcutta,

Fig. 1—Structure formula of Be and Tiron complex
India: metal contents of diet in ppm dry weight: Cu, 10; Mn, 33; Zn, 45; Co, 5) and drinking water ad libitum. Animals were given beryllium (50 mg/kg as beryllium nitrate dissolved in distilled water) i.m. once only. Animals received normal saline (4 mL/kg) by the same route and served as controls. The beryllium exposed animals were divided into various groups followed by chelation therapy for three consecutive days. Necropsy was performed 1, 3 and 7 days after the last treatment.

The groups were divided as follows:

- Group A- 4mL/kg normal saline.
- Group 1-50mg/kg Be(NO3)2
- Group 2-Be+111mg/kg (0.3 mM) CaNa2EDTA
- Group 3-Be+ 471mg/kg Tiron

The doses were selected as mentioned in the literature. Both the chelating agents were prepared daily in 0.9% saline and the pH was adjusted to pH 6.4 with sodium bicarbonate before administration. Twenty four hours after the final administration the rats were sacrificed under light anesthesia for collection of liver and blood. Blood was withdrawn by puncturing the retro-orbital venous sinus in heparinized tubes and standard techniques were employed to determine haemoglobin percentage (Sahli’s acid haematin method), blood sugar, protein, alkaline phosphatase, transaminases.

Small pieces of the liver were fixed in aqueous Bouin’s and embedded in paraffin wax. Haematoxylin and eosin stained sections were observed under microscope.

Beryllium was estimated in liver and blood using atomic absorption spectroscopy (AAS) following wet acid digestion with concentrated nitric acid.

The data were subjected to statistical analysis in order to determine the least significant difference and the variance ratio by one way analysis of variance [ANOVA].

Biochemical assay

The profile of haematological parameters showed duration dependent response after the administration of beryllium nitrate. There were no significant change in hemoglobin percentage at day 1 post exposure of beryllium nitrate, however, the percentage decreased at later durations. The activity of serum transaminases were significantly elevated one day after exposure to beryllium. A linear elevation was observed reaching peak value at day 7 post exposure of toxicant. Treatment with EDTA did not prevent increase in the activities of aspartate amino transferase and alanine amino transferase significantly. Tiron was very effective in recouping the values at all the durations showing maximum recoupment at later durations. Result reveals that the blood sugar level decreased linearly showing maximum depletion after 7 days in beryllium treated animals. A similar pattern of change was observed for serum alkaline phosphatase. At 7 days post exposure the serum alkaline phosphatase decreased significantly. This decrease in the level of blood sugar and serum alkaline phosphatase was

<table>
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<tr>
<th>Group</th>
<th>Treatment</th>
<th>Days</th>
<th>Hb%</th>
<th>blood sugar</th>
<th>Protein</th>
<th>ALP</th>
<th>AST</th>
<th>ALT</th>
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<tr>
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<td>control</td>
<td></td>
<td>15.5</td>
<td>102.8</td>
<td>41.2</td>
<td>207.8</td>
<td>70.56</td>
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<td>13.3</td>
<td>79.4</td>
<td>36.8</td>
<td>193.2</td>
<td>85.8</td>
<td>48.0</td>
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<td>37.5</td>
<td>198.0</td>
<td>79.9</td>
<td>36.7</td>
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<td>14.0</td>
<td>98.2</td>
<td>37.9</td>
<td>206.2</td>
<td>74.9</td>
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<td>33.4</td>
<td>181.1</td>
<td>98.5</td>
<td>59.9</td>
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<td>77.0</td>
<td>36.5</td>
<td>180.7</td>
<td>92.1</td>
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<td>Be+Tiron</td>
<td></td>
<td>14.8</td>
<td>85.8</td>
<td>38.8</td>
<td>192.4</td>
<td>85.9</td>
<td>49.3</td>
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<td></td>
<td>10.8</td>
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<td>150.5</td>
<td>106.7</td>
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<td>Be+Tiron</td>
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<td>13.8</td>
<td>79.7</td>
<td>37.2</td>
<td>180.4</td>
<td>91.8</td>
<td>57.0</td>
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<tr>
<td>LSD</td>
<td></td>
<td></td>
<td>2.18</td>
<td>14.4</td>
<td>8.5</td>
<td>33.6</td>
<td>11.6</td>
<td>7.8</td>
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<td>F (Variance ratio)</td>
<td>7.44*</td>
<td></td>
<td>15.2*</td>
<td>1.4*</td>
<td>4.0*</td>
<td>14.4*</td>
<td>32.7*</td>
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Significance of difference among various groups was evaluated by one way analysis of variance (ANOVA) *F=P < 0.05 comparison between two groups.

a-Group A vs group 1, b—group 1 vs group 2, c—group 1 vs group 3.

LSD—Least significant difference, ALP—alkaline phosphatase, AST—aspartate aminotransferase, ALT—alanine aminotransferase.
protected by the treatment with Tiron at all three durations, i.e. one, three and seven, however, calcium di sodium EDTA was not as effective as Tiron at 7 days regimen (Table 1). Table 2 shows maximum retention of metal was observed in liver at later days, while low concentration was detected at earlier duration. Chelation therapy was found to be effective but comparatively low level of toxicant was detected with Tiron.

Histopathology (Fig. 2A-F)

Liver of control rat showed normal features (A). Single administration of beryllium nitrate showed pathological lesions after 1, 3 and 7 days of exposure. The changes were more significant at 7 days regimen. At day one chord arrangement was slightly disturbed as a result of which irregular canaliculi were observed having debris. The nuclei also showed varied appearance some were darkly stained while others assumed star-shaped appearance. Hepatocytes in

![Histopathology Images](image1)

Fig. 2—(A) Photomicrograph of control rat liver showing typical morphological architecture. (B) After 1 day of beryllium nitrate exposure hepatocytes showing vacuolated cytoplasm and nuclei has star shaped appearance. (C) With the treatment of beryllium nitrate, note significant degeneration along with hypertrophy in hepatocytes after 7 days. (D) With Tiron treatment chord arrangement of the hepatocytes was normal after 1 day. (E) After 7 days of Tiron treatment, note regenerative changes marked by binucleated cells with normal kupffer cells. (F) CaNa2EDTA treatment produced damaged hepatocytes with severe perinuclear vacuolization. [X400]
Table 2—Effect of i.m. administration of Be(NO₃)₂ (50 mg/kg once only) followed by chelating agents for 3 consecutive days on beryllium concentration in liver and blood of adult female rats. n=5, Values are expressed in μg/g

<table>
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<tr>
<th>Treatment</th>
<th>Days</th>
<th>Liver**</th>
<th>Blood***</th>
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<tr>
<td>Control</td>
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<td>0.50</td>
<td>0.90±0.05</td>
</tr>
<tr>
<td>Be(NO₃)₂ per se</td>
<td></td>
<td>13.00*</td>
<td>12.46±1.1#</td>
</tr>
<tr>
<td>Be+CaNa₂ EDTA</td>
<td>1</td>
<td>10.22</td>
<td>10.26±0.87</td>
</tr>
<tr>
<td>Be+Tiron</td>
<td>3</td>
<td>11.79*</td>
<td>11.34±0.91*</td>
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<tr>
<td>Be(NO₃)₂ per se</td>
<td></td>
<td>16.30*</td>
<td>14.98±1.26#</td>
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<tr>
<td>Be+CaNa₂ EDTA</td>
<td>3</td>
<td>11.79*</td>
<td>11.34±0.91*</td>
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<tr>
<td>Be+Tiron</td>
<td>3</td>
<td>8.80±0.50*</td>
<td>9.54±0.65#</td>
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<td>Be(NO₃)₂ per se</td>
<td></td>
<td>22.30*</td>
<td>17.99+0.2#</td>
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<tr>
<td>Be+CaNa₂ EDTA</td>
<td>7</td>
<td>14.46*</td>
<td>13.34±1.4*</td>
</tr>
<tr>
<td>Be+Tiron</td>
<td>3</td>
<td>11.72*</td>
<td>11.40+1.0*</td>
</tr>
<tr>
<td>LSD</td>
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<td>4.39</td>
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<tr>
<td>F(Variance ratio)</td>
<td></td>
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</tbody>
</table>

** Significance of the difference among various groups was evaluated by one way analysis of variance (ANOVA) *F=P<0.05 comparison between two groups. ’—Group a vs group 1, ’—group 1 vs group 2, ’—group 1 vs group 3. LSD—Least significant difference.

*** P Values vs respective control # P<0.05, P values vs Beberyllium nitrate treated group* P<0.05

"—group a vs group 1, ’—group 1 vs group 2, ’—group 1 vs group 3

Discussion

Present investigation reveals severe alterations and duration dependent changes in various haematological and histological parameters after the beryllium exposure. A significant fall was observed in haemoglobin percentage. Macrocytic anemia, reported due to beryllium poisoning was attributed to a slow down in the synthesis of hemoglobin resulting in a consequent decrease in the haemoglobin content of the erythrocyte. Hypoglycemia from toxic dosage of beryllium salt has been reported, due to the reduced glycogenesis through inactivation of phosphoglucomutase, hexokinase and many other key enzymes involved in carbohydrate metabolism. Liver damage is implicated as a factor of importance in beryllium poisoning. Delayed removal of exogenous glucose due to liver insufficiency is the way by which toxic doses of beryllium may affect blood glucose level causing a concomitant rise in blood lactic acid. Beryllium inhibits the serum protein contents, several possibilities can be explored for explaining these results. Bulk of the circulating beryllium binds with plasma-globulin because of its apparent protein binding property and forms stable beryllium and protein complex which is transported to various organs. Thus a large amount of beryllium is accumulated in liver and causes damage. The impaired functioning of the liver which is a major source of protein synthesis and metabolism, may also diminish the plasma protein level. Serum AST and ALT are excellent markers of liver damage caused by exposure to toxic substances. Rise in serum transaminases activity as observed in the present study may be due to phagocytosis and necrosis of general had angular growths and developed perinuclear vacuoles (B). The Kupffer cells were swollen to some extent. However, at 7 days regimen includes significant degenerative changes were seen (C), which includes vacuolization of centrilobular hepatocytes, deformed nuclei with increased clumped chromatin. Focal degeneration was observed with debris inbetween the hepatocytes. As a result of heavy vacuolation the cytoplasmic granules were shifted towards the periphery the remaining nuclei showed vesicular appearance with eccentric nuclei and were surrounded by large, rounded vacuoles. Hepatic blood vessels showed dilatation, Pyknotic nuclei were observed alongside deformed sinus. Lipidolises was also seen.

Tiron therapy provoked considerable recoupment chord arrangement was maintained and lysed nuclei were comparatively less (D&E). Granulation and vacuolation was less. Calcium disodium EDTA treatment also showed protective effect. The chord arrangement was maintained when compared to beryllium per se group, however, perinuclear vacuulation was intense at some places (F). Hypertrophy of hepatocytes as observed in beryllium per se group was not seen.
liver. Such disturbances in membrane integrity is critically linked with the intracellular metabolic states and may later cause submicroscopic membrane lesion with consequent enhanced enzyme leakage. Inhibition in the activity of serum alkaline phosphates encountered during beryllium toxicity is mainly due to the displacement of magnesium ion with the beryllium ion Mg$^{2+}$ is an important factor for maintaining the activity of alkaline phosphatase. In vivo and in vitro studies have suggested that beryllium always competes with Mg$^{2+}$ and competitive inhibition results in the lowering of alkaline phosphatase activity. Inhibition may also be due to the formation of insoluble phosphate which further interferes with the absorption of phosphate in the intestine. Tiron and calcium disodium EDTA successfully restored the values throughout the experimental group when beryllium was injected. These studies were further confirmed by several pathological changes in the liver after beryllium exposure. These histological changes may be due to deposition of this compound in liver and also due to the diffusion of the colloidal compound of beryllium which may be localized in the kupffer cells into the adjacent parenchyma leading to hepatic necrosis. Liver picture showed marked improvement with both the chelates, however, with Tiron regenerative changes were more significant.

A limited number of chelating agents have been tried by several authors against beryllium intoxication. A favorable effect has been demonstrated for ethylenediamine bis-isopropyle phosphoric acid (EDPPA), Aurine tricarboxylic acid (ATA), ferritin, Ethylene glycol bis-tetraacitic acid (EGTA)\cite{11,12,14}, Shukla et al reported the efficacy of Tiron and CaNa$_{2}$EDTA in preliminary study.\cite{9}

Present study which compares both the chelates clearly revealed that Tiron offered better protection than CaNa$_{2}$EDTA against beryllium toxicity. The efficacy of Tiron to mobilize beryllium and restore the altered biochemical parameters may be attributed to the available binding sites and stability constant of the metal chelator complex formed. Beryllium has a heavy tetrahedral geometry. Its coordination number is four. Two molecules of Tiron may replace their hydrogen atoms and bind to beryllium with its oxygen atom thereby forming a stable complex (Fig. 1). In Tiron the donor groups are so placed so as to interact very strongly with the toxicant group such as -OH, -COOH, -SH, -NH, are present in such excess that atleast one group remains free, after the agent is saturated with the metal. Antidotes circulate in the blood stream without causing much depletion of essential metal concentration. EDTA has been reported to have renal toxicity and depletes essential metals like Zn, Cu, Fe, Co, and Mn, from the body.\cite{31} The ortho-diphenolic chelate structure of Tiron, forms water soluble complexes and the toxicity of these complexes is less than that of the metal ion they contain, Tiron is a superior antidote to any of the EDTA type structures examined as survival rate of animals receiving multiple injections of Tiron were enhanced over those receiving other chelates\cite{32,33}. Further the LD$_{50}$ of Tiron is significantly higher than that of other chelates.\cite{33} The present study has clearly shown that Tiron is the more effective chelator than CaNa$_{2}$ EDTA in the prevention of beryllium intoxication. However, no previous information on the clinical use of Tiron in the therapy of beryllium intoxication is available. Therefore, further investigations are required before the possible use of this compound in clinical beryllium poisoning is suggested.

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