Sub-Lethal and Teratogenicity Action of Bromadiolone and Chlorophacinone Anticoagulant Rodenticides on Albino Rats

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Abstract: Anticoagulant rodenticides have been used in controlling rodents in Egypt and all over the world. The aim of this work was to study the effect and LD₅₀ values of Bromadiolone (Broma) or Chlorophacinone (Chloro) on some biological aspects of the albino rat. The LD₅₀ value was estimated as 1.12 mg/kg for Broma and 6.31 mg/kg for Chloro. Results revealed that the Pregnancy period significantly increased under the following treatment order: Broma > Chloro > Broma / Chloro. All treatments showed drastic weight reduction for fetus when their mothers were dissected after 9 and 18 gestational day. Also similar responses were recorded at 2, 5 and 10 weeks age. The time to eye open and to hair coat were delayed significantly for all tested treatments. There were significant reductions in all tested treatments in the number of newborn. The time to death of new born rat ranged from 3.87 to 27 days with the application of Chloro and Broma, respectively. The histological studies on liver showed dilation of central and portal vein, congestion with lymphocytic infiltration and necrosis, while in kidney showed degenerative changes with necrobiosis in the lining epithelial cell of renal tubules, congestion in the glomerular tuft of the hypertrophied glomeruli, necrosis in lining epithelial cells and degeneration in the renal tubules. Biochemical studies showed increasing in enzymes.

Key words: Anticoagulants toxicity • Teratogenicity • Rodenticide • Bromadiolone • Chlorophacinone • Albino rats

INTRODUCTION

Anticoagulant rodenticides are the largest group of pesticides used for control of harmful rodents. Anticoagulant rodenticides are classified depending on their chemical structure into 2 main groups: hydroxycoumarin and indandione rodenticides [1]. The action mechanism of hydroxycoumarin and indandione anticoagulant rodenticides is identical, which yields similar clinical manifestations, hematological alterations or abnormalities and treatment schedule, regardless of the preparation group [2]. Anticoagulant rodenticides are often the cause of accidental poisoning of domestic animals. In addition, their use has the potential to cause environmental damage through poisoning of wildlife that feed on the baits or on rodents that have consumed and accumulated the poison in the body [3]. Many cases of oral intoxication both in humans [4] and animals [5, 6] had been reported. From the literature, only rare cases of human fetal anomalies were recorded regarding the developmental toxicity of coumarins especially warfarin [7, 8]. In addition, few studies were recorded in rats and mice about warfarin [9, 10] and bromadiolone [11] teratogenicity. As a result of the mammalian toxicity of anticoagulant rodenticides, the present work aims mainly to investigate the teratogenic effects and to examine the extension of these effects using sub-chronic oral LD₅₀ of bromadiolone and chlorophacinone on the fetus development and new born of the albino rat (Rattus norvegicus).

MATERIALS AND METHODS

Materials: Anticoagulant Rodenticides: Two anticoagulant rodenticides were used in this study, bromadiolone (Broma) and chlorophacinone (Chloro) obtained from KZ pesticides company, Egypt.
Bromadiolone
Chemical Class: Coumarin anticoagulant

Chlorophacinone
Chemical Class: Indandione anticoagulant

Tested Animals: Albino rats (*Rattus norvegicus*) were obtained from culture of Experimental Animals, Egyptian Organization for Biological Products and Vaccine, Helwan, Egypt. Rats were individually caged, acclimatized under laboratory conditions; each female was examined for 2 regular estrus cycles prior to initiation of the study to ensure the fertility and estrus cycle regularity of females. Every two females were caged with one fertile male overnight and checked for presence of vaginal plugs on the following morning at 7.00 a.m. The day of finding a vaginal plug was designated as zero day of pregnancy, assuming coitus took place at night of mating [12].

Methods:

**Determination of Oral LD₅₀ Values:** Serial different doses of Broma and Chloro active ingredient as mg/kg body weight were prepared and suspended in distilled water. Ten adult female rats, caged individually, were used for each dose level and were fastened for about 12 hours before treatment. Each dose was administrated by gastric intubations. A parallel control test was conducted using plain distilled water. Mortality percentages were recorded up to 28 day after treatment. LD₅₀ values were calculated by using probit transformation table designed by Weil [13] and simplified formula given by Horn [14]. Y = y + b (x - x) where x is the calculated lethal dose LD₅₀ and Y = 5 to calculate LD₅₀ y was calculated according to the percentages of mortalities and x was calculated according to the doses levels, while b = Σxy - xΣy / Σx² - x²x.

The Teratogenic Effect of Broma and Chloro on Pregnant Females and Their Fetuses: 50 pregnant female rats were divided into five groups, each group consisted of ten females their weight about (120 ± 5) gram:

**Group 1:** Ten pregnant females served as control.
**Group 2:** Ten pregnant mothers received orally ¹/₁₀ LD₅₀ (0.1122 mg/kg) of Broma in the third day of pregnancy.
**Group 3:** Ten pregnant mothers received orally ¹/₄ LD₅₀ (0.2805 mg/kg) of Broma in the third day of pregnancy.
**Group 4:** Ten pregnant mothers received orally ¹/₁₀ LD₅₀ (0.631 mg/kg) of Chloro in the third day of pregnancy.
**Group 5:** Ten pregnant mothers received orally ¹/₄ LD₅₀ (1.5775 mg/kg) of Chloro in the third day of pregnancy.

Newborns from the previous treated mothers after delivery were used for determining the biological aspects.

The Biochemical and Histological Effect of Broma and Chloro on Albino Rat Females: 50 female rats were divided into five groups, each group consisted of ten females receiving the same doses of Broma and Chloro as mentioned in the previous experiment their weight about (120 ± 5) gram. Blood samples were taken for biochemical studies and organs were preserved for histological studies.

Preparation of Blood and Organs:
**Blood:** Blood samples were collected after 7 days of treatment from each rat by retro-orbital sinus puncture in clean tube containing trisodium citrate solution (10%) as an anticoagulant. Each tube contains one ml. citrate solution/one ml. blood. Blood was centrifuged at 3000 r.p.m for 15 minutes. The supernatant plasma was collected and kept under freezing condition (- 20°C) until used.

**Organs:** Liver and Kidney organs autopsy were collected 7 days after treatment from rats in clean tube containing formalin (10%) and kept until used.

Determination of Biochemical Compounds:
Determination of enzyme activities were determined calorimetrically according to Reitman and Frankel [16] for Aspartate transaminase (AST) and Alanine transaminase (ALT), [17, 18] for Cholesterol level, [19] for Total Bilirubin level, [20] for Alkaline Phosphatase (ALP) level, [21] for Creatinine level and [22] for Urea level.
**Histological Findings:** Histological effect of Broma and Chloro on Liver and Kidney of albino rats were studied according to Banchroft et al. [23].

**Statistical Analysis:** Statistical analysis was carried out using ANOVA one way test [15].

**RESULTS**

Results in Table 1 indicated that using of ¼ $LD_{50}$ and ½ $LD_{50}$ of bromadiolone (Broma) or chlorophacinone (Chloro) increased the gestation period and related positively to the used doses; Broma ¼ followed by Broma ½, Chloro ¼ and Chloro ½ recording 23.4, 23, 22 and 21.5 days, respectively. Meanwhile, the number of fetuses per dam decreased significantly and was related positively to the used doses; Broma ¼ and Chloro ¼ which showed the highest decrease with 2.67 and 4.67 fetuses, respectively followed by Broma ½ then Chloro ½ compared to the control with 8.6 fetuses. Drastic effects in the weight reduction of fetuses which their mothers were treated after 9th and 18th day of the zero day pregnancy were observed. The highest decrease was recorded by Broma ¼ $LD_{50}$ followed by Chloro ¼ $LD_{50}$, then Broma ½ $LD_{50}$ while the minimum decreases found at the treatment by Chloro ½ $LD_{50}$ compared to the control at the 9th day with 0.49, 0.57, 0.62, 0.73 and 0.84 g and at the 18th day with 4.15, 4.7, 4.93, 5.15 and 5.69 g, respectively (Table 1). Body weight reduction of new born after treating mothers with ¼ $LD_{50}$ Broma and Chloro at 2nd, 5th and 10th week was recorded. Data in Table 1 showed 14.22 and 15.56 g at 2nd week, 32.7 and 35.32 g at 5th week, 57.88 and 62.88 g at 10th week compared with 17.7, 36.52 and 68.58 g at the same periods with control group respectively. On the other hand, new born of treated mothers with ¼ $LD_{50}$ of Broma and Chloro were died before obtaining their weights. The period to eye open and hair coat time were delayed significantly by Broma ¼ and Chloro ½ compared to the control group. The delay to eye open and fully hair coat of new born was 18.7 and 16.7 days compared with control 13.4 days for eye opening, while the fully hair coated time were 11.1 and 10.7 days compared with 7.5 days for control group, respectively. New born of treated mothers with ¼ $LD_{50}$ of Broma and Chloro were died before obtaining their weights. Number of new born and days to death of new born showed significant reduction when treated with Broma ¼ or ¼ $LD_{50}$, Chloro ¼ or ¼ $LD_{50}$ and compared with control. Broma ¼ and Chloro ¼ caused complete death of new born through the first week. While the lowest mortality rates was reported when using Chloro ½ recording 5.8 new born followed by Broma ¼ with 5.1 new born compared to 7.9 new born with the control.

**Biochemical Response:** Significant increases in the levels of Alanine transaminase (ALT) enzyme compared to control (11 IU/L) presented in Fig. 1&2 when females were treated with the two rodenticides at the sub lethal doses ¼ $LD_{50}$&¼ $LD_{50}$. The maximum increase observed when treated with Broma ¼ with 28.45 IU/L followed by Chloro ¼ with 21.68 IU/L then Broma ½ with 20.53 IU/L while the minimum increase was noticed with Chloro ½ recording 17.83 IU/L at the seventh day. Significant increase in the level of Aspartate transaminase (AST) enzyme compared to control (21.53 IU/L) was found in Fig. 2 when females were treated with the two rodenticides at the sub lethal dose ¼ $LD_{50}$. The same trend in Fig. 1 was observed when females were treated at ½ $LD_{50}$. The maximum increase was observed when treated with Broma ¼ with 41.35 IU/L followed by Chloro ¼ with 34.25 IU/L, then Broma ½ with 34.1 IU/L, while the minimum increase was noticed with Chloro ¼ recording 17.7, 36.52 and 68.58 g at the same periods with control group respectively.

<table>
<thead>
<tr>
<th>Fetus</th>
<th>Weight of fetus (g) of treated mothers at</th>
<th>Body weight (g) at</th>
<th>Days to eye</th>
<th>Fully haired</th>
<th>Number</th>
<th>Days to</th>
<th>Rate of weight</th>
</tr>
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<tbody>
<tr>
<td>Treatment</td>
<td>Gestation period</td>
<td>Number of fetus in horns</td>
<td>9th day</td>
<td>18th day</td>
<td>2nd week</td>
<td>5th week</td>
<td>10th week</td>
</tr>
<tr>
<td>Broma ¼</td>
<td>23.4</td>
<td>2.67</td>
<td>0.49</td>
<td>4.15</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Chloro ¼</td>
<td>22.5</td>
<td>4.67</td>
<td>0.57</td>
<td>4.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Broma ½</td>
<td>23.0</td>
<td>5</td>
<td>0.62</td>
<td>4.93</td>
<td>14.22</td>
<td>32.7</td>
<td>57.88</td>
</tr>
<tr>
<td>Chloro ½</td>
<td>22.0</td>
<td>6.4</td>
<td>0.73</td>
<td>5.15</td>
<td>15.56</td>
<td>35.32</td>
<td>62.88</td>
</tr>
<tr>
<td>Control</td>
<td>21.5</td>
<td>8.6</td>
<td>0.84</td>
<td>5.69</td>
<td>17.7</td>
<td>36.52</td>
<td>68.58</td>
</tr>
<tr>
<td>L.S.D. (0.05)</td>
<td>0.527</td>
<td>1.716</td>
<td>0.072</td>
<td>0.483</td>
<td>1.122</td>
<td>1.497</td>
<td>2.356</td>
</tr>
</tbody>
</table>

Broma=bromadiolone, Chloro=chlorophacinone, g=gram, d=day
Fig. 1: Effect of ($1/_{10}$ LD$_{50}$ values) of bromadiolone and chlorophacinone on liver function albino norway rat R norvegicus.

Fig. 2: Effect of ($1/_{4}$ LD$_{50}$ values) of Bromadiolone and chlorophacinone on liver function albino norway rat R norvegicus.

Fig. 3: Effect of ($1/_{10}$ LD$_{50}$ values) of bromadiolone and chlorophacinone on biochemical abnormalities of albino norway rat R norvegicus.

Fig. 4: Effect of ($1/_{4}$ LD$_{50}$ values) of bromadiolone and chlorophacinone on biochemical abnormalities of albino norway rat R norvegicus.

28.98 IU/L at the seventh day. The level of alkaline phosphatase enzyme (ALP) recorded in control (68.85 IU/L) was higher than those recorded in both rodenticide treatments. Significant decrease in the level of ALP enzyme was found when females were treated with the two rodenticides at sub lethal dose $1/_{4}$ LD$_{50}$. The same trend was observed when females were treated at $1/_{10}$ LD$_{50}$ after 7 days. The maximum decrease was observed when treated with Broma $1/_{4}$ recording 31.39 IU/L with reduction percentage of 54.41 % compared to the control, then Chloro $1/_{4}$ recording 36.28 IU/L with reduction percentage of 47.30 % followed by Broma $1/_{10}$ recording 44.02 IU/L with reduction percentage of 36.06 %, while the minimum decrease was noticed at Chloro $1/_{10}$ recording 47.29 IU/L with reduction percentage of 31.32 % after seven days.

Significant increase in plasma bilirubin at the seventh day of Norway rat females treated with broma and chloro $1/_{4}$ LD$_{50}$ recording 0.91 and 0.84 mg/dl compared to 0.65 mg/dl with the control (Fig. 4), while there was no significant increase observed with Broma and Chloro $1/_{10}$ LD$_{50}$ (Fig. 3). Broma $1/_{4}$ LD$_{50}$ caused the maximum damage for the kidney functions of the treated rats according to creatinine level, meanwhile Chloro $1/_{10}$ LD$_{50}$ was the less injury treatment after 7 days of treatment recording 0.87 mg/dl. Also the represented data showed that Broma was more harmful to kidney than Chloro for the whole tested doses ($1/_{4}$ and $1/_{10}$ LD$_{50}$ values) after 7 days of treatment. On other words the gained figures could be arrange it descending according to creatinine level after the seven days as follows: Broma $1/_{4}$ (1.68) mg/dl > Chloro $1/_{4}$ (1.41) mg/dl > Broma $1/_{10}$ (1.08) mg/dl > Chloro $1/_{10}$ (0.87) mg/dl > control (0.58) mg/dl (Fig 3&4). The obtained results revealed that both rodenticides induced an increase in urea level in plasma of treated rats when measured 7 days after treatments compared with control (0.16 mg/dl). Data revealed that, there was significant increase in the level of urea in females treated with Broma and Chloro at the sub lethal dose $1/_{4}$ LD$_{50}$ at seventh day while with $1/_{10}$ LD$_{50}$ there was no significant increase (Fig. 3&4). According to cholesterol level in plasma of treated rats when measured 7 days after treatments and compared with control value (2.43 mg/dl), the results showed that the highest increase achieved with
Fig. 5: Histological changes of albino rat liver according to sub-chronic LD₅₀ application of anticoagulant rodenticide groups.

a. Degenerative change in the hepatocytes (d). (H&E x64).  
b. Necrosis in the hepatocytes at the Centro lobular area associated with dilatation in the central vein (cv). (H&E x80).  
c. The hepatocytes (d) showed degenerative change. (H&E x80).  
d. Inflammatory cells infiltration in the portal area associated with degeneration in the hepatocytes (d). (H&E x64).

Fig. 6: Histological changes of albino rat kidney according to sub-chronic LD₅₀ application of anticoagulant rodenticide groups.

a. Edema in the perivascular area of the blood vessels (v) and inflammatory cells infiltration (o). (H&E x80).  
b. Swelling in the lining endothelium of the glomerular tuft (g). (H&E x80).  
c. Swelling and proliferation in the endothelial cells lining the glomerular tuft (g). (H&E x160).  
d. Swelling in the lining epithelium of the tubules (S) and showing congestion in the glomerular tuft (g). (H&E x160).

Fig. 7: Histological study of untreated albino rat liver and kidney.

a. No histopathological alteration and normal histological structure of the central vein (cv) and surrounding hepatocytes (n). (H&E x64).  
b. No histopathological alteration and the normal histological structure of the glomeruli (g) and tubules (t) at the cortex (H&E x64).

Broma ¼ LD₅₀ was 3.27 mg/dl followed by Chloro ¼ and treated albino rats, Rattus norvegicus with different doses of Broma and Chloro anticoagulant rodenticides, induced, degenerative changes with necrobiosis (the physiological death of a cell and can be caused by certain conditions such as abasiophilia, erythema or the presence of a tumor) in the lining epithelial cell of renal tubules, congestion in the glomerular tuft of the hypertrophied glomeruli, necrosis in lining epithelial cells, degeneration in the renal tubules (Fig. 3&4). The histopathological studies for the untreated albino rats showed normal histological structures of liver and kidney (Fig. 7).

**Histopathological Findings:** Histopathological studies in the livers induced by different doses with Broma and Chloro anticoagulant rodenticides on treated albino rats, Rattus norvegicus showed dilation of central and portal vein, congestion with lymphocytic infiltration and necrosis at treatment periods (Fig. 5). Regarding to the histological changes in the kidneys of treated albino rats, Rattus norvegicus with different doses of Broma and Chloro anticoagulant rodenticides, induced, degenerative changes with necrobiosis (the physiological death of a cell and can be caused by certain conditions such as abasiophilia, erythema or the presence of a tumor) in the lining epithelial cell of renal tubules, congestion in the glomerular tuft of the hypertrophied glomeruli, necrosis in lining epithelial cells, degeneration in the renal tubules (Fig. 6).


**DISCUSSION**

The present study demonstrated the embryo-toxicity and teratogenicity of two anticoagulant rodenticide groups, Bromadiolone (Broma) and Chlorophacinone (Chloro) in the progeny of rats treated orally at ¼ and ½ LD₅₀ values during the period of organogenesis. Exposure of rats to Broma and Chloro at the two tested doses retarded fetal development and induced embryotoxic and new born effects as evidenced by decreased fetal weight, reduced number of new born per dam and time to eye opening and to hair coat. The number of dead fetuses was increased in exposed rats compared with the control group. Similar results were previously reported by Feteih et al. [9] and Howe and Webster [10] in rats after exposure to coumarin during the organogenesis and fetal periods. In addition, Twigg and Kay [11] confirmed the adverse effect of Broma rodenticide on the breeding performance of house mice (Mus domesticus) at sub-lethal doses equating to between 20% and 70% of the acute LD₅₀ per feed. Teratogenic interference in the period of organogenesis, when most major organs and body regions are being established, is usually related to (major) structural anomalies. The fetal period is characterized by histogenesis and functional maturation; the influence of a teratogen in this period may cause growth retardation or functional disturbances [24]. Abadi et al. [25] and Ageno et al. [26] confirmed that coumarin derivatives including warfarin pass the placental membrane and has the potential to cause bleeding in the fetus and teratogenicity when used in early pregnancy. Coumatetralyl might be accumulated in the fetuses due to excessive transfer from maternal blood through placenta to fetus. Therefore, the impaired fetal physiology in Coumatetralyl-treated group resulting in embryo-and fetotoxic effects might be due to coumatetralyl accumulation as also seen in warfarin and other coumarin derivatives [27]. In addition, disturbances in the placental microcirculation and hemorrhages induced by this rodenticide may alter placental function and impair embryonic and fetal development. Consequently, the placenta may be directly involved in many instances of early spontaneous abortion and, fetal death and intrauterine growth retardation [28]. The earliest concepts of the pathogenesis in the congenital anomalies found after in utero exposure to coumarin derivatives were based on the main clinical effects, the prolongation of blood clotting time. It was suggested that deformities in the new born were caused by micro hemorrhages and subsequent scaring and calcification [29]. In light of these results, the early embryonic deaths noticed in female rats exposed to Racumin rodenticide most probably resulted from micro hemorrhages and consequently modification of the uterinelining function before arrival of the embryo and/or micro hemorrhages and subsequent scaring and calcification after implantation. The action mechanism of hydroxycoumarin and indandione anticoagulant rodenticides is identical (Fig. 8), which yields similar clinical manifestations, hematological alterations or abnormalities and treatment schedule, regardless of the preparation group [2]. Anticoagulant rodenticides inhibit the recycling of vitamin K₁, a cofactor of primary importance for postribosomal carboxylation (activation) of blood clotting factors II (prothrombin), VII (proconvertin), IX (Christmas factor) and X (Stuart prower factor), by the enzyme vitamin K-dependent carboxylase, maintaining the active form of vitamin K [30, 31]. By the enzyme vitamin K-dependent carboxylase, the active vitamin K is transformed into an inactive epoxide that is thereafter reconverted into vitamin K (vitamin K quinone), by the enzyme vitamin K epoxide reductase. In the next step, the vitamin K reductase converts vitamin K quinone into vitamin K hydroquinone that is integrated again in the carboxylation cycle of blood clotting factors II, VII, IX and X. Then the enzyme vitamin K reductase converts vitamin K quinone into vitamin K hydroquinone that enters once again the carboxylation cycle of blood clotting factors II, VII, IX and X [32]. Anticoagulant rodenticides inhibit vitamin K epoxide reductase, resulting in a lack of active vitamin K. This mechanism contributes to blood clotting factors (II, VII, IX and X) that are not carboxylated and remain nonfunctional [1, 33] because anticoagulant rodenticides do not block these factors, their concentrations in blood decrease about 12-24 h after the intoxication coinciding with the first massive bleeding episodes [30, 31, 34]. The observed generalized edema and hemorrhages in the obtained fetuses from treated female rats in the present study may be attributed to the disturbed coagulation and clotting mechanisms induced by both applied anticoagulant rodenticides.

In the histology of treated animals with Broma, kidney exhibited necrosis, degeneration and accumulation of toxic metabolic debris in the renal glomerular and tubular cortex region with swelling cells [35]. In Mus musculus after 40-48 hrs exposure to anticoagulant rodenticide Broma, showed the presence of blood in the urine, this may be due to necrosis and degeneration of renal cortex as seen in the histological preparations. Wolf and Carlton [36] also reported in Swiss albino mouse, occurrence of acute cortical tubular necrosis due to the
administration of 2 Bromoethylamine hydrobromide. Buckley [37] reported degeneration of distal tubules, necrosis and tubular dilatation in mice when treated with anticoagulant rodenticides. Severity of this type of rodenticides on kidney degeneration increases with respect to time and dose. In the present study the histological study was in harmony with these investigations showing swelling in the lining epithelium of the tubules, edema, inflammatory of cells and congestion in the glomerular tuft. In liver sections, there was a clear multifocal cytoplasmic vacuolations, necrosis and accumulation of toxic debris. This pathological condition progressively increased from 6, 12, 24 and 48 hrs [35]. This was in conformity with the toxicity studies of C.I. pigment red, conducted at National institute of Environmental health sciences, Birmingham, USA on the liver, kidney and spleen which were the target tissues in mice, showed haematopoietic cell proliferation in both liver and spleen [38]. It was also positive to iron pigments in the spleen. The present study was also in agreement with histological findings of Buckley et al. [39] and Shooba [40]. According to the effect of anticoagulants on biochemical abnormality. The obtained results are in harmony with those reported by Binev et al. [41], Boermans et al. [42], James et al. [43] and Kohn et al. [44], who stated the presence of Hypoproteinemia, hypoalbuminemia, hyperglycemia, bilirubinemia, high urea concentration and high activities and quantities of alanine transferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and gamma glutamyltransferase. Hemorrhages and hematomas in the renal parenchyma and bilateral hydronephrosis [45], bleedings, hematomas and dystrophic damage of liver parenchyma [46] may be the main causes for the abnormality of kidney and liver functions and enzymes in the present investigation. It can be concluded that bromadiolone (Broma) and chlorophacinone (Chloro) embryotoxic and teratogenic rodenticide when administered during the organogenesis period. This embryotoxicity can be produced even at low doses which may not manifest any alarming clinical signs of toxicity in exposed dams. Therefore, it is recommended that farm animals and mammalians must be kept away from gaining access to either Broma or chloro- containing rat baits or even Broma or chloro- poisoned rats specially during pregnancy.

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