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ORIGINAL ARTICLE

Difethialone intoxication induced haematological changes in Indian desert gerbil, *Meriones hurrianae* (Jerdon)

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ABSTRACT
Difethialone, a second-generation anticoagulant rodenticide has been found to be highly effective against warfarin resistant strains of rats and mice. Assessment of the toxicity of Difethialone was done on haematological indices of Indian desert gerbil, Meriones hurrianae. The exposure of acute median lethal dose (LD_{50}-0.28 mg/kg body wt.) of the rodenticide leads to anemic reaction in gerbils, with a varying response at different time intervals of 2, 4, 6, 8 days post treatment on both the sexes. There was a significant decrease in haematological values of Erythrocyte count, PCV, haemoglobin per cent and MCHC, and a significant increase in MCV and MCH values. This decrease was observed to be time and dose dependent. Altered blood parameters show that the treated gerbils suffered from pernicious anaemia. The test anticoagulant rodenticide exerted poisoning symptoms manifested in the form of lack of anorexia, sluggishness, bleeding claws of the fore and hind limbs, traces of blood in the ears, nose and sometimes in the conjunctiva of the eye and general paralysis. The results could provide baseline information for the toxicity of Difethialone in rodent control.
Key words: Difethialone, Haematological indices, LD_{50}, pernicious anaemia, toxicity.

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INTRODUCTION

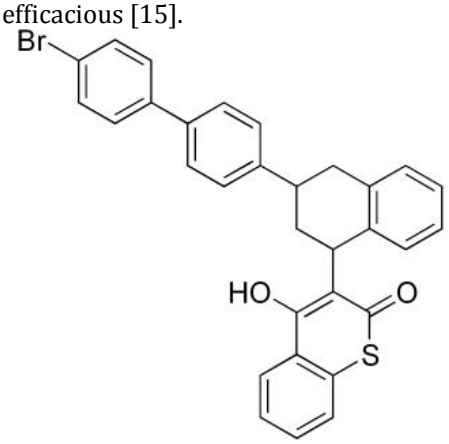
Rodent pest control campaigns are largely dependent on anticoagulant rodenticides which are much selective in their activity. The traditional first-generation anticoagulants are multi-dose rodenticides. Development of bait shyness and resistance to the first generation anticoagulant rodenticides led to the discovery of a new series of hydroxycoumarin derivatives. These are the second generation anticoagulant rodenticides, also sometimes referred to as superwarfarins [1] and are far more toxic than first generation anticoagulants. They are applied in bait formulations and can effectively poison a rodent after only a single dose [2]. Blood parameters are widely used in mammals to assess any alteration in their physiology. 4-Hydroxycoumarins and indandiones are the two major categories of the oral anticoagulants [3] and function by inhibiting the prothrombin formation, thus interfering in the process of blood coagulation. Murray and Tseng [4] demonstrated delayed clotting of whole blood as a diagnostic tool in free-ranging raptors. Rattner *et al.* [5] validated the utility of blood clotting time in captive avian wildlife as a biomarker to assess anticoagulant rodenticide exposure.

Coumarin derivatives are used widely and cause severe injury to vascular permeability, leading to massive haemorrhages and resultant rapid death of rodents [6,7,8]. Difethialone is a second generation anticoagulant which belongs to a new class of chemicals, hydroxy-4 benzo thiopyranones formed by the replacement of one oxygen atom by a sulphur atom in hydroxy-5 coumarin. Arora *et al*., [9] reported 100 per cent mortality in *Rattus rattus* with three different concentrations of Difethialone viz. 0.0025, 0.00125 and 0.00375 per cent, both in no-choice and choice feeding tests after 24, 48 and 78 hours baiting period. The anticoagulant activity of Difethialone has been observed on several rodent species in field and laboratory conditions. Difethialone (0.0025%) yielded commendable control success and effectively cleared the population of *M. meltada*, *M. booduga* and *Bandicota bengalensis* in growth stages of sugarcane [10]. Difethialone manifested itself to be the most important anticoagulant rodenticide in about 63% (19/30) of the poisoning cases and is equally or more potent threat to rodent-eating raptors than

brodifacoum [11]. In a survey done by Murray [12,13] on raptors and submitted to wildlife clinic in Massachusetts, US, a similar change in detection of difethialone and multiresidue cases was observed in general. 100% mortality was recorded with difethialone admixed cereal-based bait at a concentration as low as 0.0013%. Any increase in toxicant concentration was found to have no effect on the mortality pattern. No marked difference was observed in bait acceptance and palatability ratio among various concentrations [14].

MATERIAL AND METHODS

Toxicant used - Difethialone, a second generation anticoagulant rodenticide is the only representative of the benzothiopyranone family having a sulphur atom in external position in aromatic ring, due to which it is reserved in liver and becomes efficacious [15].



Formula of Difethialone

Experimental design - 120 adult gerbils were collected from the harvested fields. Acclimatization of gerbils to laboratory conditions was done for two weeks prior to the experiment. The animals were maintained on a pelleted diet and water ad libitum. Calculated single dose of Difethialone (LD_{50}-0.28 mg/kg body wt.) was administrated to healthy adult gerbils of both the sexes. The average weight of gerbils was between 80-120 gms. Oral forced feeding of the dose was done with the aid of a stomach gavage needle. The vehicle control was carried out with the poison free carrier, propylene glycol. The animals were autopsied on 2^{nd}, 4^{th}, 6^{th} and 8^{th} days after the administration of dose.

Blood collection - Blood was obtained from the heart of anesthetized gerbils selected randomly (16). Cardiac puncture was done and blood was drawn in sterilized 1cc syringe with 24 gauge needle. The sample blood was collected in separate heparinized vials for males and females. The blood was stored in refrigerator until analysis.

Haematological analysis - The blood samples were used to determine the red blood cell count on improved Neubauer haemocytometer, packed cell volume estimation and haemoglobin content estimation was done using standard procedures.

MCH, MCHC, and MCV are components of red cell indices (parameters indicating size and haemoglobin content of red blood cells) that have conventionally been used to aid in the differential diagnosis of anaemia (17). Standard haematological indices MCV, MCH and MCHC were calculated from the RBC count, haemoglobin content and the volume of packed red blood cells.

The haematological parameters were analysed for statistical significance using ANOVA and the significant differences checked at $P < 0.05$. The toxic effects of Difethialone are depicted graphically for all haematological parameters for treated animals in comparison to vehicle control groups.

RESULTS AND DISCUSSION

Toxicity symptoms - High toxicity of test article, Difethialone was noted. The administration of the rodenticide resulted in the onset of poisoning symptoms within 2-3 days. The animals were quite active on the first day of poisoning but become sluggish progressively with loss in weight. Loss of appetite may be attributed to the sickness caused by the poison. A prominent feature observed in all the treated gerbils was uneasiness and pulmonary distress. Traces of blood were observed in the ears, nose and conjunctiva of the eye of the treated animals. Bleeding from the claws of fore and hind limbs was a conspicuous feature of Difethialone poisoning. Excessive urination and frequent passing of the faecal matter was observed in intoxicated animals as compared to the control animals.

Autopsy of dead animals revealed various affected organs. The liver turned pale progressively and numerous yellowish blisters appeared on its surface. Ballooning of alimentary canal was observed. Subcutaneous haemorrhage was seen. Internal hemorrhage occurred and un-coagulated blood was observed in the visceral cavity at various places.

Non- significant difference was observed at all autopsy intervals between Normal control and vehicle control groups in all haematological parameters.

Erythrocyte count - The RBC count decreased gradually at all autopsy intervals in males (day2 $P > 0.05$; day4 $P < 0.01$; day6 and 8 $P < 0.001$) and females (day2 $P < 0.01$; day 4, 6 and 8 $P < 0.001$). (Fig. 1 and 2). The decline in erythrocyte count was found to be differential and was more in the females as compared to males. The decreased RBC count due to Difethialone intoxication may be attributed to the inhibition of erythropoiesis in the bone marrow due to anticoagulant activity. Ineffective erythropoiesis due to anticoagulant poisoning causes death of the developing red blood cells in the bone marrow. The poison enters into the RBCs through their cell membrane and leads to their destruction in albino mice treated with Bromadiolone, a second generation anticoagulant rodenticide [18]. Revathi and Yogananda, [19] reported remarkable decrease in Hb, RBC, WBC and platelet count in the Bromadiolone treated *Mus musculus*.

Reduced RBC count may also be due to the direct effect of the anticoagulant which causes continuous haemorrhage and subsequent blood loss.

Haemoglobin content - The haemoglobin present in the erythrocytes in Difethialone treated gerbils was found to decrease considerably. Progressive decrease in haemoglobin content was noticed post treatment in males (day2 $P < 0.05$; day4 and 6 $P < 0.01$; day8 $P < 0.001$) and females (day2 $P < 0.05$; day 4, 6 and 8 $P < 0.001$). (Fig. 3and 4) The decline in haemoglobin concentration may be due to rapid destruction of erythrocytes. The anticoagulant enters the red blood cells through their cell membranes which become fragile and rupture during their passage through the fine capillaries. The excessive loss of blood due to severe haemorrhage results in the influx of fluids from the body reserves causing the dilution of blood resulting in declined haemoglobin level. The chemical injury caused due to Difethialone poisoning is most likely responsible for the destruction of RBCs thus destroying haemoglobin. Similar effects were reported with Brodifacoum [18]. Significantly decreased haemoglobin and RBC count was observed following exposure of Bromadiolone to rats, *Bandicota bengalensis* (20). Administration of anticoagulant rodenticide bromadiolone to house mice, *Mus musculus* reduced the blood platelets count and resulted in degeneration of liver and kidney [19].

Haematocrit - Treatment with Difethialone recorded measurable decline in packed cell volume of males (day2 $P > 0.05$; day4 and 6 $P < 0.01$ land day8 $P < 0.001$) and females (day2 $P > 0.05$; day 4, 6 and 8 $P < 0.001$). (Fig. 5 and 6) The anticoagulant has a direct effect on the cell membranes of RBC and brings about their destruction. Low rate of RBC production leads to a reduced erythrocyte count. The per cent haematocrit is affected by this.

The anticoagulant rodenticides affect the cardiovascular system of the target animals. Coumarin and its derivatives are known to reduce the plasma levels of prothrombin and factors VII, IX and X, all formed by the liver. To verify Anticoagulant Rodenticides exposure in animals they were analysed in plasma by coagulation test [21]. Administration of an effective dose of anticoagulant warfarin decreases the coagulant activity of blood by 50 per cent by the end of 12 hours. The mechanism of action of anticoagulant rodenticides is the inhibition of Vitamin K, a precursor in the synthesis of blood-coagulation factors. The onset of clinical symptoms varies from 3 to 5 days of the first exposure of the rodenticide due to the existing reserves of Vitamin K in target animals necessary for the synthesis of blood-clotting factors [22]. The coagulation process is not blocked immediately instead it awaits the consumption of prothrombin and other blood clotting factors which are already present in the plasma.

Haematology of various rodent species after the administration of different anticoagulants has been studied by several workers. Experimental intoxications of dogs with bromadiolone, a second generation anticoagulant rodenticide, showed altered clinical and haematological parameters in dogs followed by reduced haematocrit (23,24). Acute intoxication with Bromadiolone anticoagulant rodenticide led to significant changes in haematological parameters (erythropaenia, reduced haematocrit) and prolonged prothrombin time (25).

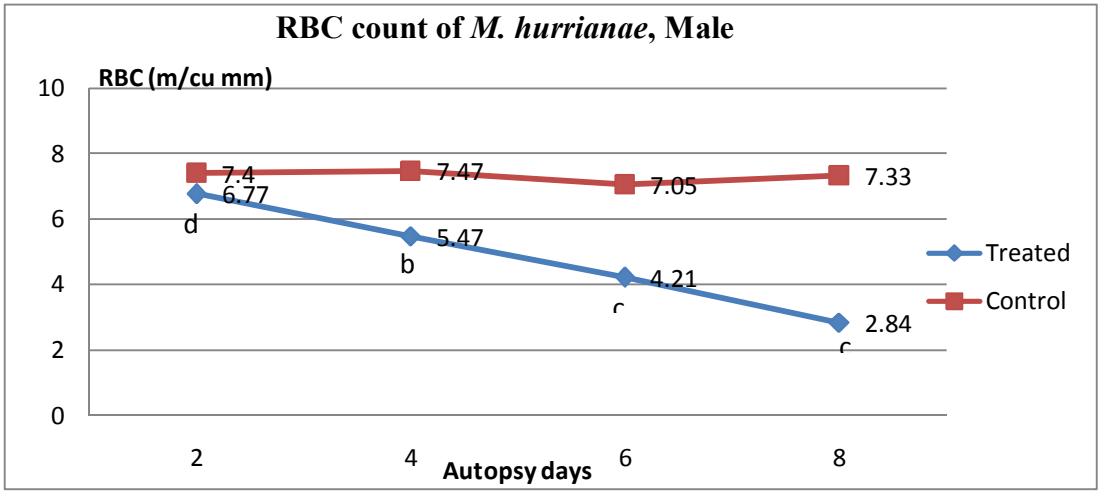
Difenacoum (4-hydroxycoumarin) the newest second generation anticoagulant rodenticide can lead to sub-lethal effects based on its half-life and toxicity data and has the same mode of action as warfarin [26,27].

Mean Corpuscular Volume (MCV) - The MCV values increased sharply in males (day2 $P > 0.05$; day4 $P < 0.01$; day 6 and 8 $P < 0.001$) and a gradual statistical increase was recorded in females from 2nd to 8th day post treatment (day2 $P < 0.05$; day4, 6 and 8 $P < 0.001$). (Fig. 7 & 8)

Mean Corpuscular Haemoglobin (MCH) - Rise in MCH values was observed in both males (day2 and 4 $P < 0.05$; day6 and 8 $P < 0.001$) and females (day2 $P < 0.05$; day4 $P > 0.05$; day6 and 8 $P < 0.001$) at all autopsy intervals. (Fig. 9 and 10)

Mean Corpuscular Haemoglobin Concentration (MCHC) - MCHC values declined after dose administration on all autopsy intervals in males (day2 $P > 0.05$; day 4 $P < 0.05$; day6 $p < 0.01$; day8 $P < 0.001$) and females (day2 $P > 0.05$; day4, 6 and 8 $P < 0.001$). (Fig.11 and 12).

MCH, MCV and MCHC as measures of CBC can be used for the estimation of anaemia. Intoxication with Difethialone results in increase in MCV and MCH while decline was noticed in MCHC in both the sexes. The deficiency of RBC in blood causes anaemia which may be due to excessive blood loss or increased destruction of erythrocytes (haemolysis). After severe haemorrhage, the lost plasma is restored within a short time but not the RBC, thereby causing anaemia. During erythropoiesis, several maturing factors like vitamin B_{12} and folic acid are essential. These are stored in the liver and transported to bone marrow. In the absence of any of them, the RBC count is greatly reduced. Slow production of fragile and oversized RBCs called megaloblasts occurs in the bone marrow. Such a condition leads to pernicious (megaloblastic) anaemia. Difethialone toxicity results in altered blood parameters of the test species leading to internal and external haemorrhage and finally death.



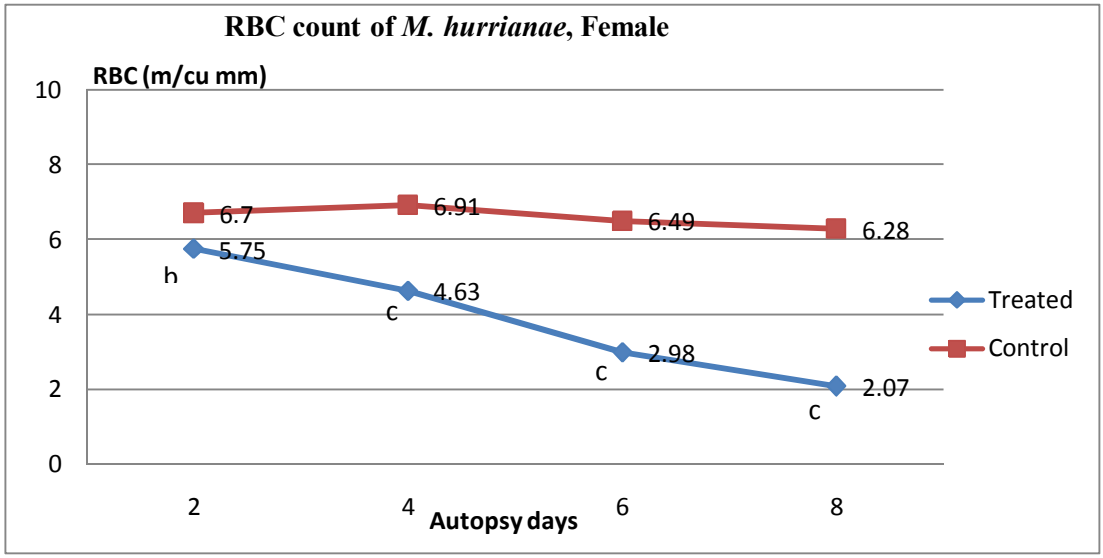
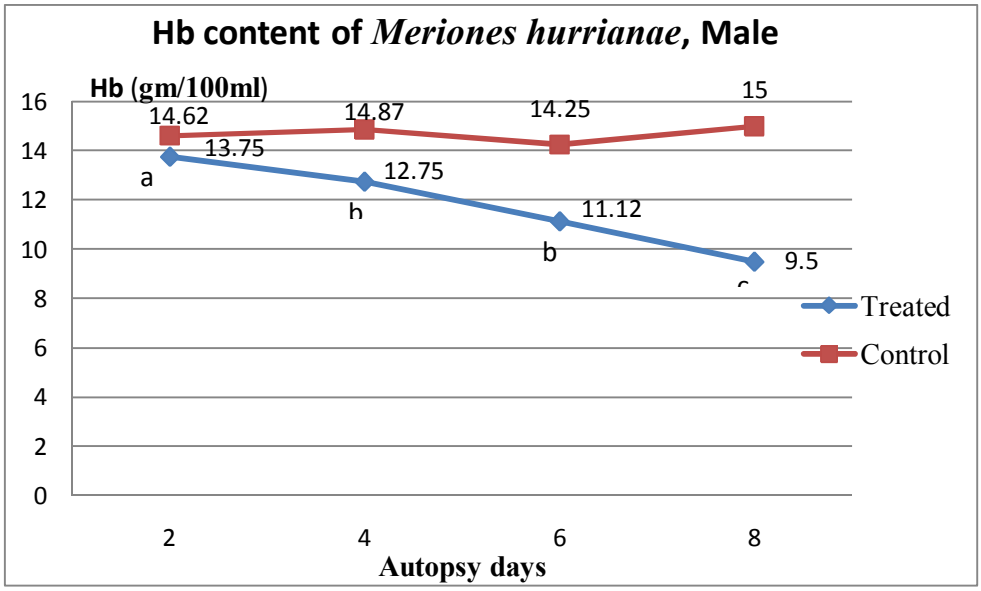


Fig. 1 & 2: Toxic effects of Difethialone on RBC count of *Meriones hurrianae*, Male & Female, Dose level: 0.28 mg/kg body wt. b: $P < 0.01$; c: $P < 0.001$; d: $P > 0.05$



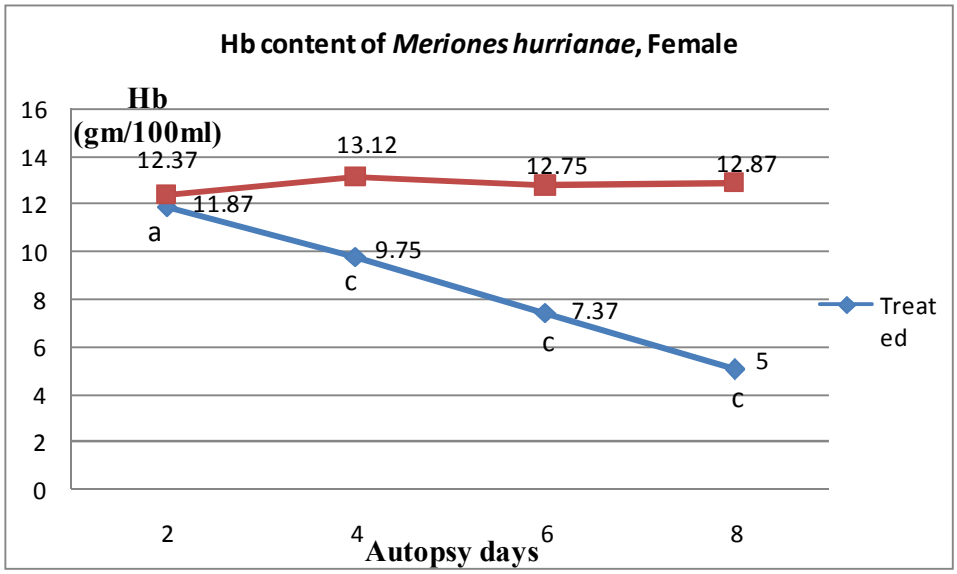
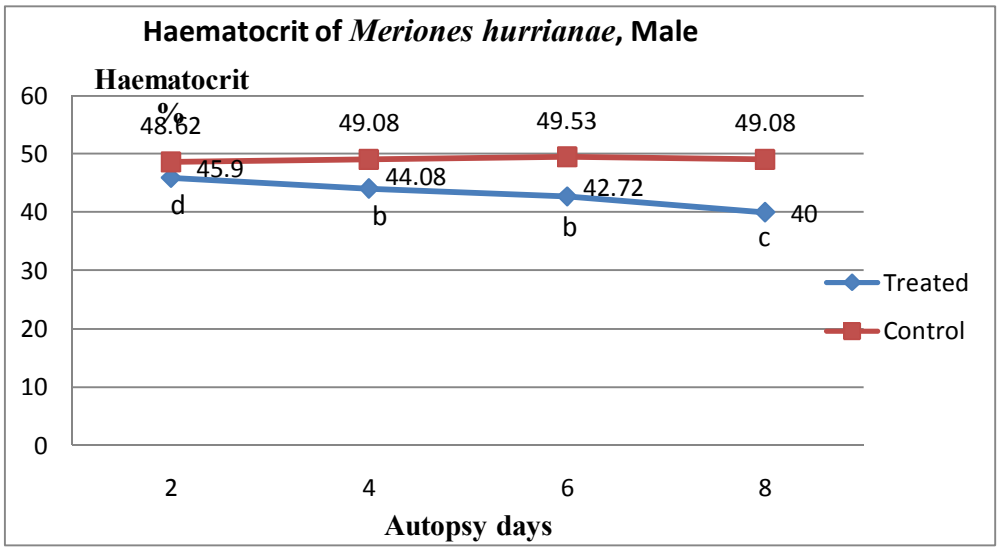


Fig. 3 & 4: Toxic effects of Difethialone on Haemoglobin content of *Meriones hurrianae*, Male & Female, Dose level: 0.28 mg/kg body wt. a: P < 0.05; b: P < 0.01; c: P < 0.001



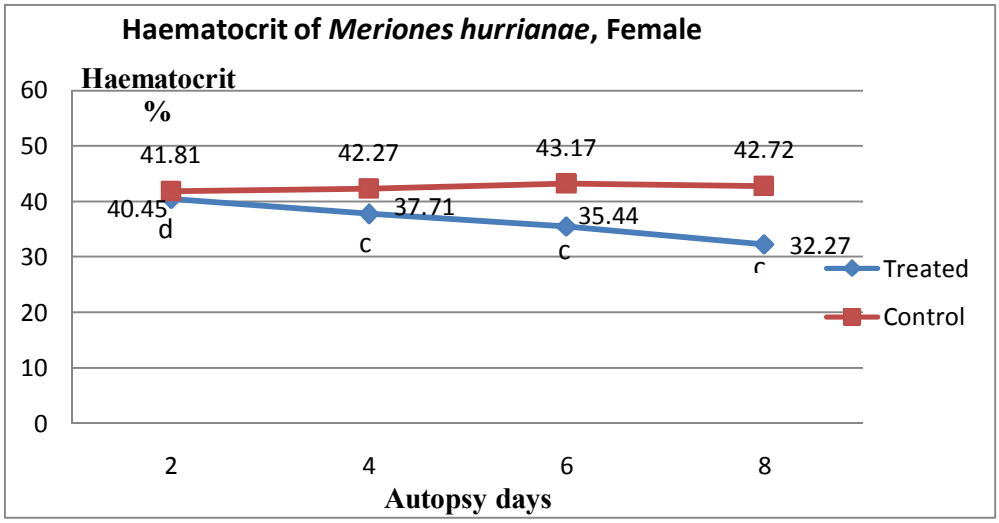
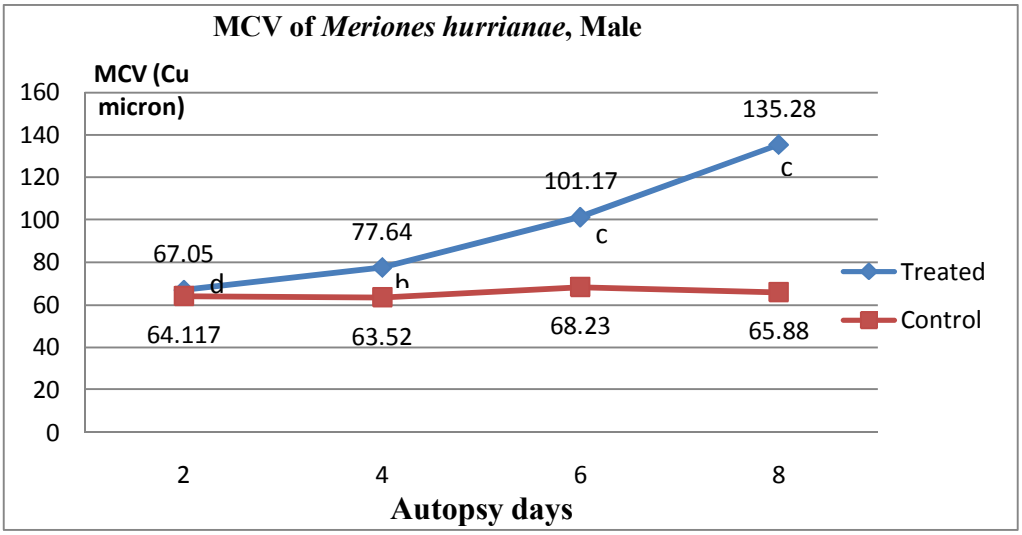


Fig. 5 & 6: Toxic effects of Difethialone on Packed cell volume of *Meriones hurrianae*, Male & Female, Dose level: 0.28 mg/kg body wt. b: P < 0.01; c: P < 0.001; d: P > 0.05



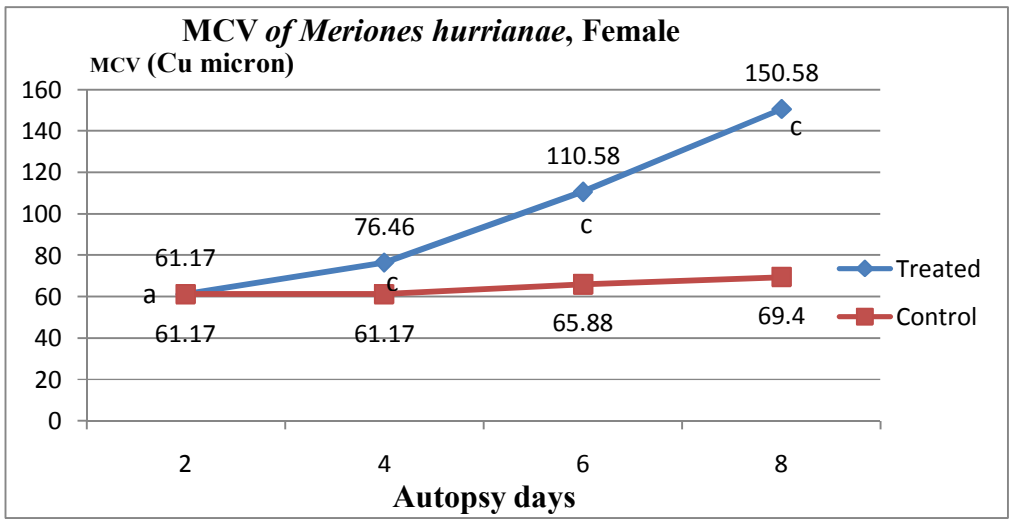
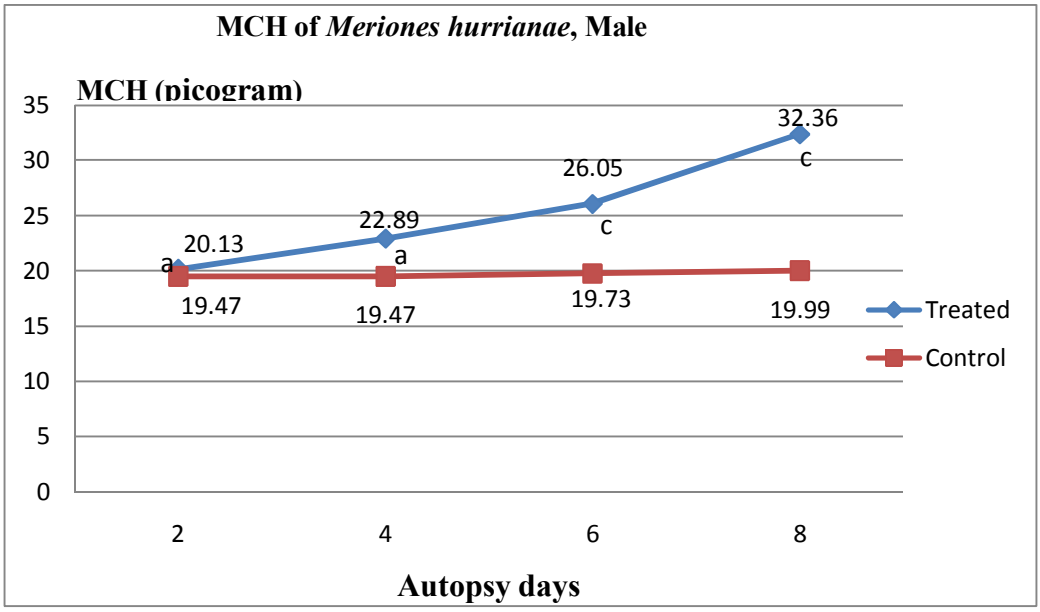


Fig. 7 & 8: Altered MCV of *Meriones hurrianae*, Male & Female due to Difethialone Toxicity, Dose level: 0.28 mg/kg body wt. a: P < 0.05; b: P < 0.01; c: P < 0.001; d: P > 0.05



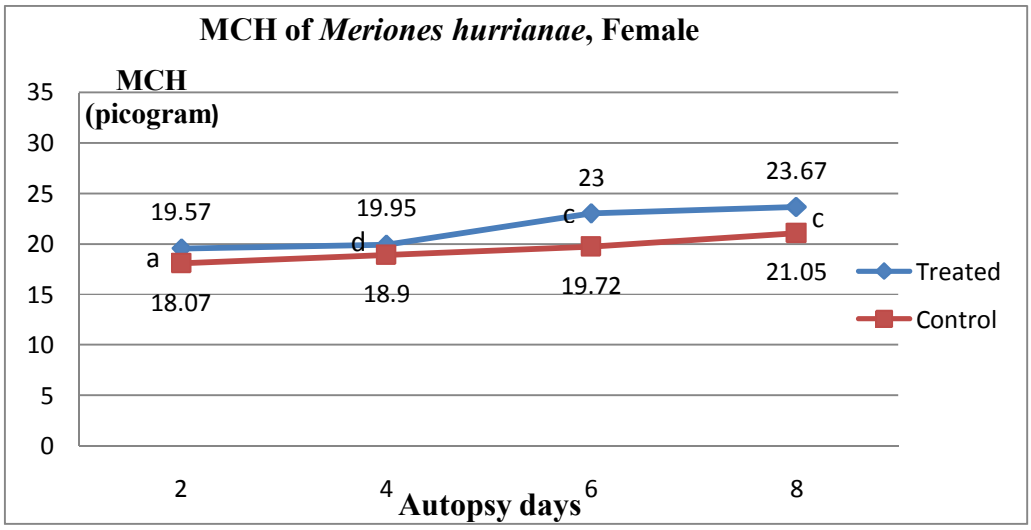
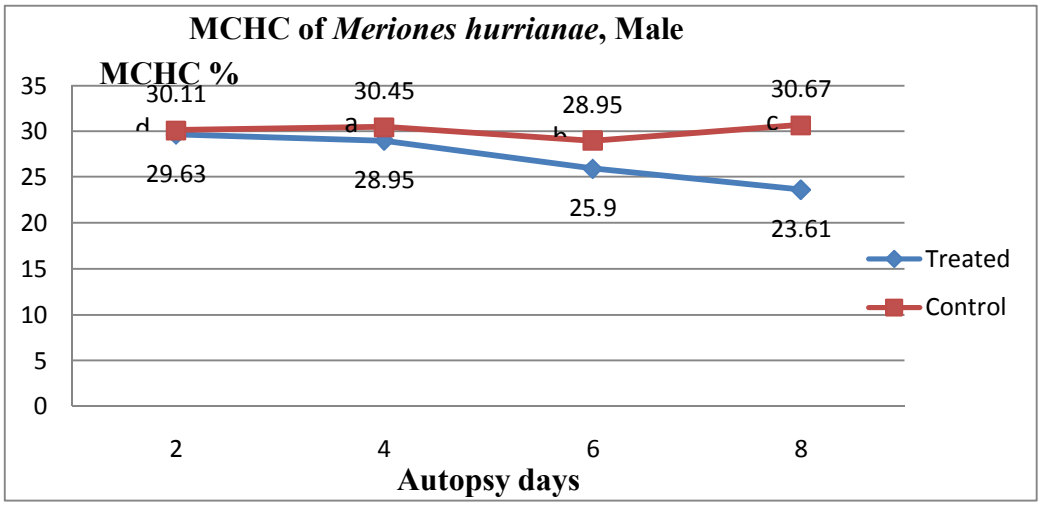


Fig 9 & 10: Altered MCH of *Meriones hurrianae*, Male & Female due to Difethialone Toxicity, Dose level: 0.28 mg/kg body wt. a: P < 0.05; b: P < 0.01; c: P < 0.001; d: P > 0.05



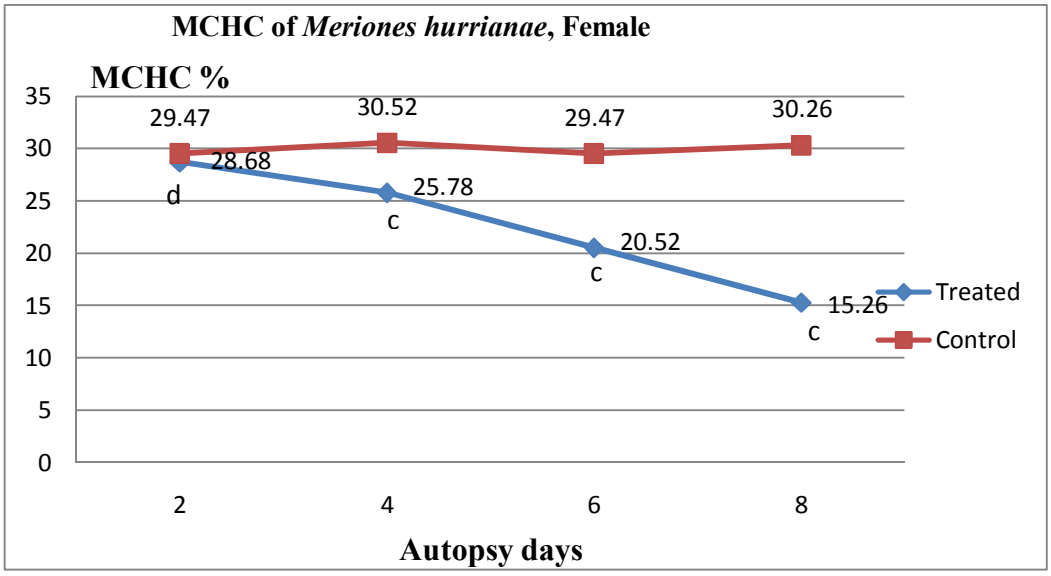


Fig. 11 & 12: Altered MCHC of *Meriones hurrianae*, Male & Female due to Difethialone Toxicity, Dose level: 0.28 mg/kg body wt. a: $P < 0.05$; b: $P < 0.01$; c: $P < 0.001$; d: $P > 0.05$

CONCLUSION

A very low concentration of the toxicant (LD_{50} – 0.28 mg/kg body wt.) is effective in the control of gerbils. Till date, no cases of resistance have been reported with Difethialone, hence it seems to be a potent second generation single dose anticoagulant toxicant effective in controlling *Meriones hurrianae* under laboratory conditions. Elementary haematology tests are potent screening methods for determining efficacy of anticoagulant rodenticide toxicosis in rodents. Hence, the study could be taken up for the control of field rodent species.

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