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THE EFFECTS OF MEFENAMIC ACID ON THE BLOOD HAEMOGLOBIN OF THE LIZARD, *UROMASTIX HARDWICKII*

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This study deals with the effect of 7.1mg/day, 10.5mg/day and 14mg/day doses of mefenamic acid administered for 12 days to three groups of *Uromastix hardwickii* respectively. Individual blood samples were obtained from the anterior abdominal vein and hemoglobin content was determined. The hemoglobin in test was 5.1g/100ml compared to 8.0g/100ml of controls in experiment I and its amount remained almost similar in the case of experiment II, whereas, 4.5g/100ml was observed of test compared to 8.0g/100ml of their counterparts.

INTRODUCTION

Autoimmune heamolytic disease (AHD) is characterized by an increased destruction of the patient's own erythrocytes. The accelerated haemolysis is mediated by one or more mechanisms involving components of the immune system.

The earliest chemical description of AHD citing evidence was that of Chaufford and Vincent (1909), Widal *et al.* (1908); which can now be interpreted in terms of autoimmune mechanism.

Various abnormalities in the red cell may shorten the normal life span of 120 days. Anemia develops when marrow output of new red cells is reflected in a raised reticulocytes count which gives an indication of the severity of the process. A number of commonly prescribed drugs can induce production of both types of warm and cold antibodies which is associated with autoimmune hemolytic anemia (Schwartz *et al*., 2000). Thus studies were carried out to find out the effects of mefenamic acid on the lacertilian hemoglobin (Hb) in the lizard, *Uromastix hardwickii.*

The more common hemolysis is the removal and destruction of erythrocytes with membrane alteration by the macrophages of the spleen and liver. Circulating blood is filtered continuously through thin walled splenic cords into splenic sinusoids, a sponge like labyrinth of macrophages with long dendrite processes (Chadburn, 2000).

The destruction of RBC is characterized by increased unconjugated bilirubin, increased lactate dehydrogenase and decreased haptoglobin levels. Lactate dehydrogenase and Hb is released into circulation when RBCs are destroyed. Liberated Hb is converted into unconjugated bilirubin in the spleen or may be bound in plasma by haptoglobin. The Hb-haptoglobin complex is cleared quickly by the liver, leading to low or undetectable haptoglobin levels (Marchand *et al*., 1980).

Autoimmune hemolytic anemia is mediated by autoantibodies and further subdivided according to their maximal binding temperature. Warm hemolysis refers to IgG autoanitbodies, which maximally bind RBCs at body temperature (37^{o}C [98.6^{o}F]). In cold hemolysis, IgM autoantibodies bind RBCs at lower temperature (0^{o}to 4^{o}C [320 to 39.2^{o}F]).

When warm autoantibodies attach to RBCs surface antigens, these IgG – coated RBCs are partially ingested by the macrophages of spleen, leaving microspherocytes, the characteristic cell of autoimmune hemolytic anemia (Maedel and Sommer 1993). These spherocytes which have decreased deformability compared with normal RBCs are trapped in the splenic sinusoids and removed from circulation.

Cold autoantibodies (IgM) temporarily bind to RBC membrane and activate complement and deposit complement factor C3 on the cell surface. The C3-coated RBCs are cleared slowly by macrophages of the liver (Engelfriet *et al*., 1992).

In the present study, the effect of various doses of mefenamic acid on the Hb of the lizard, *Uromastix hardwickii* have been investigated.

MATERIALS AND METHODS

Design of experiment

There were altogether four groups of ten lizards each, one group was kept as control and the other three groups served as tests (Ahmad *et al*., 2004).

Drug administration

In experiment I each animal received 1ml suspension of 7.1 mg mefenamic acid per day for 12 days. In experiment II each test animal received 1ml suspension of 10.5 mg mefenamic acid per day for 12 days and in experiment III each test animal received 14 mg mefenamic acid per day for

12 days. For feeding tests and control animals were fed with 1ml of 5% glucose solution thrice a week.

Collection of blood

For the estimation of (Hb), blood was collected from the anterior vein (Ahmad *et al*., 2003) of control and test animals on day 6 and day 12 for each experiment.

Methods of estimation of Hb

Several methods have been described for the estimation of Hb. Sahli's acid-haematin method is less accurate than any of the methods mentioned; as the colour which develops does not become stable but begins to fade almost immediately after it reaches its peak. There are methods available with merits and disadvantages. There is little to choose in accuracy between methods employing photoelectric colorimeters; although the alkaline haematin procedure is probably less accurate than the cyanmethaemoglobin method and the oxyhaemoglobin method.

The cyanmethaemoglobin method (Drabkin *et al*., 1908) although quite accurate, but (requiring the use of the poison sodium cyanide) was adopted for this study.

Estimation of Hb

The Hb content of a solution may be estimated by several methods, viz (1) by measurement of its colour, (2) its power of combining with oxygen or carbon monoxide, (3) or by its iron content. The clinical methods are all colour or light intensity matching techniques, efficiently measuring the presence of varying proportions of inert pigments (methaemoglobin or sulphaemoglobin).

Collection of blood samples for determination of Hb

Free-flowing blood from anterior abdominal vein (Ahmad *et al*., 2004) added with few crystals of sodium citrate was used. The concentration of anticoagulant was not critical. However, the measurements can also be carried out on blood stored at 4 ^{0}C for several days; provided it does not become obviously infected. Such blood must be allowed to attain room temperature and should be mixed well before use (Ahmad *et al*., 2005).

Drabkin's cyanmethaemoglobin modified reagent method

The Hb was treated with reagents containing potassium ferricyanide, potassium cyanide and sodium bicarbonate. The ferricyanide from methaemoglobin converted into cyanmethaemoglobin by the cyanide. The reagent was prepared by dissolving 1g of sodium bicarbonate ± 0.2 g of potassium cyanide, and finally made upto liter with the addition of double distilled water.

Procedure

0.02 ml of blood was added to 5 ml of Drabkin's reagent after shaking several times and standing for 10 – 30 minutes the transmittance was observed in Beckman spectro-photometer at 540 nm and Hb in g/100ml blood was worked out from the standard cyanmethaemaoglobin curve.

Preparation of standard curve and a standard table

For reading results of all samples, a standard curve (or table) of optical density of Hb concentration in g/100ml was used. The curve was obtained by measuring the optical density of cyanmethaemoglobin standard at room temperature against a blank of cyanide – ferricyanide reagent, in the photometer to be used for subsequent haemoglobinometery. Readings of optical density were also obtained with the same standard solutions diluted with the reagent in a ration 1:2, 1:3 and 1:4 etc.

The Hb values of the solutions were translated into g /100 ml, as described above. The readings were plotted on linear graph paper using arithmetical scales, with optical density as ordinate and Hb (g/100ml) as abscissa. Since, Lambert-Beer's law is valid for cyanmethaemoglobin, the points should fall on straight line passing through the origin, to correspond the linear calibration of the photometer, indicating the correct dilution of the standard. The standard curve thus obtained facilitates corresponding the observed optical densities and working out the Hb values and a table thus prepared is always more convenient than that of graph when large numbers of measurements are to be made. The main advantages of cyanmethaemoglobin method for the determination of Hb are allowance of direct comparison with the cyanmethaemoglobin standard in case the sample is not read immediately following dilution. It has also the advantage of converting all forms of Hb to cyanmethaemoglobin except that of sulphaemoglobin.

Table 1

Hemoglobin concentration (g/100 ml) of control and test following oral administration of 7.1mg/ml mefenamic acid per individual/day at 32 ± 1^{o}C

Days	Control	Test
0	8.9 ± 0.20	8.2 ± 0.11
1	8.8 ± 0.17	8.2 ± 0.12
2	8.6 ± 0.14	8.0 ± 0.02
3	8.5 ± 0.16	8.0 ± 0.08
4	8.4 ± 0.13	8.0 ± 0.03
5	8.3 ± 0.11	7.8 ± 0.08
6	8.3 ± 0.12	7.5 ± 0.08
7	8.3 ± 0.15	7.2 ± 0.12
8	8.2 ± 0.11	7.0 ± 0.16
9	8.1 ± 0.14	6.2 ± 0.20
10	8.0 ± 0.20	6.0 ± 0.13
11	8.0 ± 0.12	5.8 ± 0.02
12	8.0 ± 0.15	5.1 ± 0.19

Each figure is the mean ± SD of 10 values

Table 2
Hemoglobin concentration (g /100 ml) of control and test following oral administration of 10.5mg/ml mefenamic acid per individual/day at 32 ± 1°C

Days	Control	Test
0	8.9 ± 0.20	9.0 ± 0.21
1	8.8 ± 0.17	8.8 ± 0.16
2	8.6 ± 0.14	8.5 ± 0.08
3	8.5 ± 0.16	8.5 ± 0.10
4	8.4 ± 0.13	7.8 ± 0.11
5	8.3 ± 0.11	7.5 ± 0.19
6	8.3 ± 0.12	7.5 ± 0.03
7	8.3 ± 0.15	6.8 ± 0.21
8	8.2 ± 0.11	6.2 ± 0.21
9	8.1 ± 0.14	6.2 ± 0.20
10	8.0 ± 0.20	5.8 ± 0.08
11	8.0 ± 0.12	5.5 ± 0.26
12	8.0 ± 0.15	5.0 ± 0.19

Each figure is the mean ± SD of 10 values.

Table 3
Hemoglobin concentration (g/100ml) of control and test following oral administration of 14.0 mg/ml mefenamic acid per individual/day at 32 ± 1°C

Days	Control	Test
0	8.9 ± 0.20	9.0 ± 0.21
1	8.8 ± 0.17	9.0 ± 0.15
2	8.6 ± 0.14	8.4 ± 0.11
3	8.5 ± 0.16	8.0 ± 0.06
4	8.4 ± 0.13	7.5 ± 0.18
5	8.3 ± 0.11	7.0 ± 0.13
6	8.3 ± 0.12	7.0 ± 0.04
7	8.3 ± 0.15	6.4 ± 0.20
8	8.2 ± 0.11	6.2 ± 0.22
9	8.1 ± 0.14	5.6 ± 0.09
10	8.0 ± 0.20	5.0 ± 0.19
11	8.0 ± 0.12	4.7 ± 0.11
12	8.0 ± 0.15	4.5 ± 0.08

Each figure is the mean ± SD of 10 values.

RESULTS

A consideration of table-1 indicates that Hb concentration is slightly reduced in controls, it was 8.9g/100 ml on day 0, 8.4/100 ml and 8.0g/100ml on day 4 and 12 respectively. However, a marked reduction in mean Hb concentration was observed from day 4 onward in animals of all test groups treated with different doses of mefenamic acid.

On day 4 the test group administered 7.1mg/ml mefenamic acid showed a difference in mean Hb concentration of 0.4g/100 ml (table 1), but the test animals given a dose of 10.5mg/ml mefenamic acid showed a fall of mean Hb concentration of 0.6g/100ml as compared to their counterpart (table 2). While the difference of mean Hb concentration was 0.9g/100 ml in test group treated with 14mg/ml mefenamic acid (table 3).

The difference of mean Hb concentration between control and test groups became more significant on day 12, i.e. 2.9, 3.0 and 3.5g/100ml for doses of 7.1, 10.5 and 14mg/ml mefenamic acid respectively.

The fall in the mean Hb concentration in test animals given the higher dose i.e. 14mg/ml of mefenamic acid for 12 days showed a greater reduction in Hb concentration in comparison to test groups administered lower doses. It was 4.5g/100ml which was almost about half to its initial concentration of 9.0g/100ml (table 3).

An observation of fig.1 indicates that mean Hb concentrations of control animals from day 0 to day 12 remained constant and though test group graphs followed the same path; but started to decline from day 4 onwards. The graph for test group given a low dose of 7.1mg/ml mefenamic acid occupied a position nearer to control in contrast to the test group administered a high dose of 14mg/ml mefenamic acid.

For each test group the mean Hb concentration, when compared and analyzed statistically with mean Hb concentrations of control showed a significant reduction ($p<0.05$) irrespective of either given a low dose or a high dose of mefenamic acid.

DISCUSSION

The catabolic pathways for Hb degradation are overloaded and there is a modest increase in unconjugated bilirubin in the blood and increased reabsorption of urobilinogen from the gut, which is then excreted in the urine in increased amounts. Bile does not appear in the urine. Since, Hb is liberated into the plasma where it is bound mainly by α – 2 globulin, haptoglobulin to form a complex too large to be lost in the urine. This degraded Hb is taken by the liver.

If all the Hb is consumed, free Hb may be lost in the urine (Uthman, 1998). In small amounts it is reabsorbed by the renal tubules where the Hb is degraded and iron stored as haemosiderin. In *Uromastix hardwickii* water is reabsorbed from the urinary bladder and nitrogenous wastes produced are excreted out as solid balls.

Drug induced hemolysis which is observed in present investigation by the decrease in Hb concentration after oral administration of mefenamic acid in doses of 7.1 mg/ml, 10.5 mg/ml and 14.0 mg/ml per individual/day at 32 ± 1°C showed that the mechanism of action may be classified into

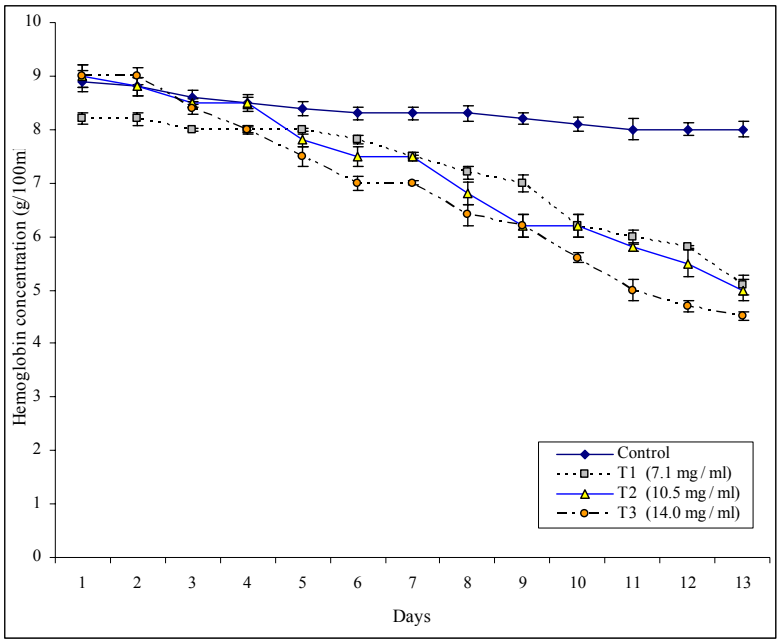

Fig.1: Hemoglobin concentration of control and test groups following administration of different doses of mefenamic acid for 12 days.

drug absorption (hapten-induced), immune complex or autoantibody (Schwartz *et al*., 2000). These IgG and IgM mediated disorders produce a positive DAT (direct antiglobin test) and are clinically and serologically indistinct from autoimmune hemolytic anemia. Statistically significant decrease in Hb concentration after mefenamic acid administration is also reported by Suhail M *et al*. (1995).

Drug probably exerts inhibition on H_2O_2 forced erythrocytic membrane lipid peroxidation. Mefenamic acid evokes catalase and glutathione peroxidase reaction with red cell H_2O_2. A number of recent studies indicate that catalase is the primary enzyme responsible for protecting the red cell from H_2O_2 (Nagababu *et al*., 2003). NSAIDs cause the activation of glutathione S-transferase. Erythrocyte catalase activity is thus increased by NSAIDs, and is involved in the antioxidative processes of erythrocytes (Orhan *et al*., 2001). These observations suggest that mefenamic acid causes functional impairment of red cell membrane leading to the release of Hb.

Hemolytic effect of drug may be due to fluid retention or an effect upon erythropoiesis. Although the exact mechanism is unknown; but it may be possible that mefenamic acid by altering a RBC membrane protein and rendering its antigen (Petz 1993), induces the production of antierythrocyte IgG antibodies and causes hemolytic anemia, thus by weakening its own backbone, through anchoring its lipid bilayer, the membrane undergoes a progressive deterioration in structure and results in spherocytes which are unable to pass through the splenic cords and are degraded and ingested by monocyte macrophage system.

The mature RBC, while biochemically complex, is a relatively simple cell that has extruded its nucleus, organelles and protein synthesizing machinery. Defects in any of the remaining component-enzymes, membrane and Hb can lead to hemolytic anemia.

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