Pharmacological Effects of Diclofenac Sodium on Some Hematological Parameters of Male Rabbits

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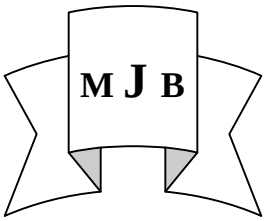

Abstract

Diclofenac sodium isa non steroidal anti inflammatory drug (NSAIDs). The present study was conducted to assess the effects of Diclofenac sodium on some hematological parameters,on male rabbits. The study was carried out in the animal house of College of Medicine-Babylon University at the period from 1 Dec. 2009 -1Mar. 2010. Twenty local male rabbits were divided in to two groups, (control and treatment).The drug was given intramuscularly once a day. Along the study period Diclofenac sodium is given for 45 days and, blood samples were obtained every 15 days for hematological study , while at day 60 blood samples are used for hematological and immunological study. As a results of this study,Diclofenac cause significant decrease ($p< 0.05$) in hemoglobin conc. and hematocrit percentage and significant increase ($p< 0.05$) in reticulocyt percentage as compared with control group. As for WBCs related parameters there is non significant difference ($p> 0.05$) in total WBCs count of all the three treated group as compared with control group. On the other hand differential WBCs count show significant decrease ($p<0.05$) in neutrophils count and significant increase ($p< 0.05$) in lymphocytes count of treated group as compared with control group. Diclofenac treated animals show erythroid hyperplasia. In conclusion Diclofenac used in this study caused anemia and neutropenia and these effects were due to immune and non immune mechanisms.

الخلاصة

دايكلوفيناك لصوديوم هو واحد من مضادات الالتهاب اللاستيرويدية، الهدف من الدراسة هو بيان تاثير دايكلوفيناك على بعض المؤشرات الدموية ونخاع العظم لذكور الارانب .تمت الدراسة في البيت الحيواني التابع لكلية الطب –جامعة بابل للفترة من ١ كانون الاول ٢٠٠٩ ولغاية ١ اذار ٢٠١٠.عشرون أرنبا من الذكور تم استخدامها في البحث قسمت إلى مجموعتين ، الأولى تمثل مجموعة السيطرة و مجموعه علاجية. تزرق بالدواء عضليا مره كل يوم لمدة خمس وأربعين يوما،و اخذ عينات الدم كل خمسة عشر يوما، لدراسة التغيرات الدموية، وفي اليوم الستين عينات الدم استخدمت لدراسة التغيرات الدموية والمناعية،و تم اخذ عينات من نخاع العظم.

توصلت هذه الدراسة إلى النتائج التالية: إن ،دايكلوفيناك الصوديوم قد سبب انخفاضا معنويا ($p< 0.05$) في تركيز خضاب الدم ونسبة الخلايا المترسبة وارتفاع معنوي ($p< 0.05$) في نسبة الخلايا الشبكية مقارنة بمجموعة السيطرة. أما فيما يتعلق بمؤشرات كريات الدم البيض فليس هناك تغير معنوي ($p> 0.05$) في العدد الكلي لهذه الكريات ، من جهة أخرى اظهر عدد كريات الدم البيض النوعي انخفاضا معنويا ($p< 0.05$) في نسبة خلايا العدلة وارتفاعا معنويا ($p< 0.05$) في نسبة الخلايا اللمفاوية مقارنة بمجموعة السيطرة. أظهرت نتائج مسحات نخاع العظم مايلي: الدكلوفيناك قد سبب زيادة في نشاط نخاع العظم .نستنتج من ذلك ان دواء الدكلوفيناك قد تسببت في فقر الدم عند الارانب خلال مدة الدراسة بسبب عوامل مناعية او غير مناعية.

Introduction

Diclofenac is a non steroidal anti-inflammatory drug belongs to phenyl acetic acid group. Diclofenac sodium has been used in human medicine for many years for the long term symptomatic treatment of rheumatoid arthritis, osteoarthritis, and soft tissue inflammation / injuries. It may also be useful for short term treatment of acute musculo-skeletal injury and dysmenorrheal pain. The daily dose varies between 50 and 150 mg/day and may be used up to 12 weeks[1,2]. Diclofenac has rapid absorption, extensive protein binding, and a short half-life 2–3 hours. There is a substantial first-pass effect, such that only about 50% of Diclofenac is available systemically. Diclofenac accumulates in synovial fluid after oral administration[3-5]. Which may explain why its duration of therapeutic effect is considerably longer than the plasma half-life. Diclofenac metabolized in the liver by a member of the CYP2C subfamily to 4-hydroxydiclofenac, the principal metabolite, and other hydroxylated forms; after glucuronidation and sulfation the metabolites are excreted in the urine (65%) and bile (35%)[6,7]. Diclofenac has analgesic, antipyretic, and anti-inflammatory activities. Its potency against COX-2 is substantially greater than several other traditional NSAIDs[8]. In addition, Diclofenac appears to reduce intracellular concentrations of free AA in leukocytes, perhaps by altering its release or uptake. The selectivity of Diclofenac for COX-2 resembles that of Celecoxib[9,10].Common adverse effects are gastrointestinal adverse effects including: abdominal pain, nausea, and dyspepsia .Cardiovascular: hypertension and heart failure due to fluid retention, minor CNS symptoms and allergic or local reaction[11,12]. Hematological adverse effects: Salama *etal*,2003[13] declare that auto antibodies and drug or metabolites dependent antibodies have been found in patients with Diclofenac induced immune hemolytic anemia. Miyamoto *etal*, 1997[14] show that Diclofenac is associated with low, but significant incidence of hepatotoxicity and bone marrow toxicity and they have been suggested that this could be due to reactive acyl glucuronide. J.S. Jurgensen *et al*, 2001[15] revealed that diclofenac or its metabolites appeared to induce immune hemolytic anemia by the production of drug–dependent antibodies plus auto antibodies in series of patients. Gutting *etal*, 2002[16] observed that diclofenac is associated with several distinct allergic and autoimmune like reaction including anaphylaxes, idiosyncratic hepatotoxicity and auto immune hemolytic anemia. Saches *et al*, 2004[17] observed that diclofenac is a widely used NSAIDS implicated as a cause of immune hemolytic anemia. Ahrens *etal*, 2006[18] stated that evidence has shown that the drug (Diclofenac) or its metabolites may lead to the production of drug-dependent antibodies and/or auto antibodies against red blood cell.

Drug- induced hematological effect.

Neutropenia: is a decrease in circulating neutrophils in the

peripheral blood it is commonly defined as a circulating neutrophil count of less than 1500.

Anemia.: can be defined as a reduction in hemoglobin concentration of blood; this is reduction usually accompanied by a fall in red cell count and packed cell volume (P.C.V.)[19]

Causes of anemia

Blood loss: excessive bleeding such as hemorrhages or abnormal menstrual bleeding.

Impaired red cell production:

Chronic illness secondary to refractory anemia: inflammatory GI/GU diseases, malignancies (cancer), arthritis, kidney or liver failure, and acute and chronic infections

Cancer therapy: surgery, radiotherapy, chemotherapy and/or immunotherapy, Infiltration (replacement) of bone marrow with cancer.

Ineffective erythropoiesis and low levels of erythropoietin (a hormone produced by the kidney {90%} and liver {10%}) which promotes red blood cell production.

Increase rate of red cell destruction and hemolysis : can be defined as those anemia's which result from an increase in the rate of red cell destruction resulting in red cell survival less than 120 days[19]. The present study aims to clarify the effects of Diclofenac sodium on selected hematological parameters namely (hematocrit value(Ht), hemoglobin concentration (Hb c), white blood cell count (WBCS count), WBCS differential count, reticulocyte count, platelet count, Clarify the effects on bone marrow by bone marrow film study.

Materials and methods

Twenty mature (local strain) male rabbits (1-2) kg weight were reared in controlled condition at animal house of College of Medicine in Babylon University at the period from 1Dec.2009 to 1Mar.2010.The rabbits were allowed two weeks to adapt the environmental condition at animal house (room temperature of about 23-25 C and light -dark cycle of 10 light: 14dark hours, concentrated food, drinking water were supplied *ad lebitum* and were kept in cages).

Drug. Drugs used in the experiment were**:** Diclofenac sodium intramuscular injection ((olfen,)® MEPHA, Germany).0.3mg /kg.

Experimental design: The experiment was designed for 60 days long through which blood samples were obtained at zero day (before start treatment) from all groups in the study, then drug was administered to the treatment group as follows for 45 day starting from day zero to the end of day 45 along which blood samples were obtained every 15 days (day 0, 15, 30, and 45): At the end of day 60 blood samples have been collected from groups for hematological and immunological study and bone marrow aspirate obtained .3 ml of blood collected by direct heart puncture at (day 0, 15, 30, 45, &60) from all groups. The blood was put in EDTA tubes of about 2.2 ml for hematological analysis.

Blood film preparation : Blood film is prepared by placing drop of blood on the center line of glass slide 1cm away from the proximal end. Then by using spreader slide the drop is spread without delay. After that the film is dried in the air and flood the slide with

Leishman stain. 2min later we add double volume of water and the slide is left for 5-7 min. Finally the slide is washed by running tab water, dried and examined under light microscope [20].

Hemoglobin concentration (Hb conc.): Determination of hemoglobin concentration is done by converting hemoglobin to cyanomethhemoglobin using drabkin reagent and read by spectrophotometer at 540 nm[20].

Hematocrit measurement: Capillary tube of 75mm in length and 1 mm in width is used. Blood is allowed to fill 2/3 of the capillary tube, and then one of the ends is sealed with clay. After that the capillary tube is centrifuged for 5 min in hematocrit centrifuge and the proportion of cells to the whole column of blood is measured by special reading device[20].

Reticulocyte count: 2-3 drops of reticulocyt stain (new methylene blue dye solution) is delivered to 75x10 mm glass tube by mean of Pasteur pipette and 2 volume of EDTA anti coagulated blood is added to the dye solution and mixed .After that the mixture is kept in 37?C water bath for 15 minutes. At the end of the 15 minutes the mixture is resuspended by gentle mixing. Finally smears are made on glass slides and examined after dryness directly using x100 oil immersion objective lens[21].

Total white blood cells count (W.B.C): Estimation of total white blood cell count has been carried out using Neubaur improved chamber slide and 2% acetic acid solution as a diluents in about 1:20 ratio of dilution to dissolve red blood cells membrane[20.22]

Differential Leukocytes Count (%): Using x40 objective lens of light microscope, different leucocytes percentages has been calculated in Leishman's stained blood films. A total of 100 leucocytes per each slide are examined to count neutrophils, lymphocytes, monocytes, eosinophiles and basophiles.

Platelet count: Estimation of platelet count has been carried out using Neubaur improved chamber slide and ammonium oxalate 10 mg/l as a diluents in about 1:20 ratio of dilution[20].

Anti globulin test (Coomb's test). This test is indicated for detection of incomplete antibodies coated erythrocytes in hemolytic anemia. It involves direct antiglobulin test (DAT) and indirect antiglobulin test (IAT).**Direct antiglobulin test (DAT)** DAT is indicated to demonstrate in- vivo attachment of antibodies to red cells. **0.5** ml of blood delivered to a glass tube and washed three times by isotonic saline. Then, 2% suspension of red cells is made. In another tube 1 drop from the suspension is placed. Then 2 drops of anti–human globulin serum AHG Coomb's serum) are added and the mixture is centrifuged for 30 second at low speed. At the end of centrifugation the tube is shacked gently and observed for agglutination by hand lens or low power objective of microscope[20].

Indirect antiglobulin test (IAT). In some cases antibodies are formed but they are of such a nature or present in small amounts that DAT test fail to detect so IAT test is carried out .IAT is used to demonstrate antibodies in

the test serum thought to have hemolytic anemia.

2 drops of test serum and 1 drop of 3% suspension of group O Rh-D positive red cell are mixed in glass tube .Then red cells are washed three times with 3ml saline per wash by using slow speed centrifuge for 30 second. After that the mixture is incubated at 37?C in a water bath for 15 minutes. Finally, 2 drops of (AHG) are added to wash cells and centrifuged after thorough mixing and observed for agglutination as in DAT[20].

Determination of the IgG and IgM protein by radial immunodiffusion plate. To examine the protein diffusion in agar gel containing a specific antibody, an immune – complex can be seen as a ring around the wells (the diameter of the ring is in proportion with the concentration of the analyzed protein. **Bone marrow aspiration:** The aspiration and study of smears of bone marrow is often used as a diagnostic procedure in the investigation of blood dyscrasias on complete blood picture. **Aspiration technique**

Iliac crest is decided as the area of aspiration. Special needle with stylate is used. The bone is entered 2-3 cm below the crest in mid-axilary line, with twisting motion and gentle pressure. Then, aspiration is started as soon as the needle seams to be rigidly held in place. After that the sty late is removed and another dry syringe is used to aspirate around 1-2ml of marrow. Leishman's stained smears are made directly and by using oil immersion objective the percentage of different nucleated cells are identified[21].

statistical analysis The data expressed as mean ± SEM unless otherwise stated. Statistical analyses were done by using ANOVA. significant difference was set at $P < 0.05$.

Results

Effects of treatment on red cell related parameters and haemostatic function.

In this study there was significant reduction ($p<0.05$) in hematocrit percent and hemoglobin concentration and significant increase ($p<0.05$) in reticulocytes percentage in group of treatment as compared with the control group, Table (1) .Study of haemostatic function through primilary investigations shows non significant difference ($p> 0.05$) in platelet count of treated group as compared with control group, Table (1).

Effects of different treatments on white blood cells related parameters.

In this study there were non significant difference ($p>0.05$) in total WBC count in treated group as compared with normal control group, Table (2) . There was significant decrease ($P<0.05$) in neutrophiles percent and significant increase ($p < 0.05$) in lymphocytes percent in treated group as compared with normal control group, Table (2). While, there were non significant difference ($p>0.05$) of eosinophils percent and monocytes percent in treated group as compared with normal control group, Table (2).

Table 1 Mean value of red cell parameters and platelet count of the treated group as compared with normal control group. *p < 0.05

Parameters	Control Mean ±SEM	Diclofenac Mean ±SEM
Hematocrit %	44.5± 1.201	39.08±1.201*
Reticulocyte %	2.36±1.119	6.766±1.119*
Hemoglobin conc.	14.4±0.4	12.02±0.4*
Platelet count cell/l	337.4±25.31	292.88±25.31

Table 2 Mean values of white blood cells parameters of treated group as compared with normal control group.

parameters	Control Mean ± SEM	Diclofenac Mean ± SEM
WBC count	5620±394.23	4750±394.23
Neutrophil %	57.8±2.538	44.36±2.538*
Lymphocyte %	32.2±2.33	49.54 ±2.33*
Monocytes %	3.5 ±0.4	5.32 ±0.4
Eosinophil %	2 ±0.5	1.64 ±0.5

*p < 0.05

Effect of Diclofenac sodium (0.3 mg/kg) on red cell related parameters in Diclofenac treated group compared with control group.

There was significant decrease (p< 0.05) in hematocrit percent and hemoglobin conc. of Diclofenac treated group as compared with control in which the lowest hematocrit percent and hemoglobin conc. at day 45, Figure (1 ,3). There was significant increase (p< 0.05) in reticulocyts percentage of Diclofenac treated group as compared with control in which the highest reticulocyts percentage at day 45, Figure (2).But, there was no significant difference (p> 0.05) in platelet count of Diclofenac treated

group as compared with control during study period.

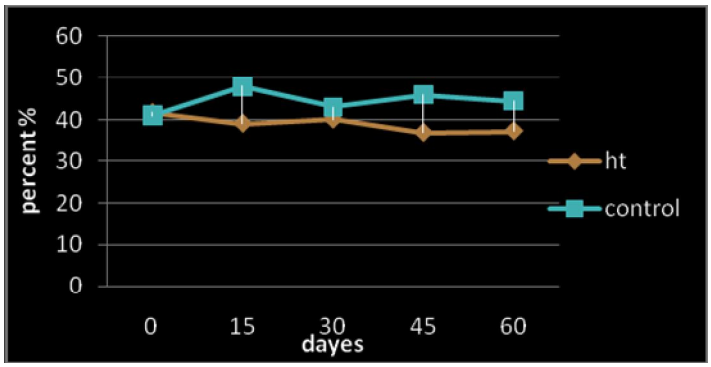

Figure 1 Mean changes in hematocrit percent Diclofenac treated group as compared with control group. ht=hematocrit

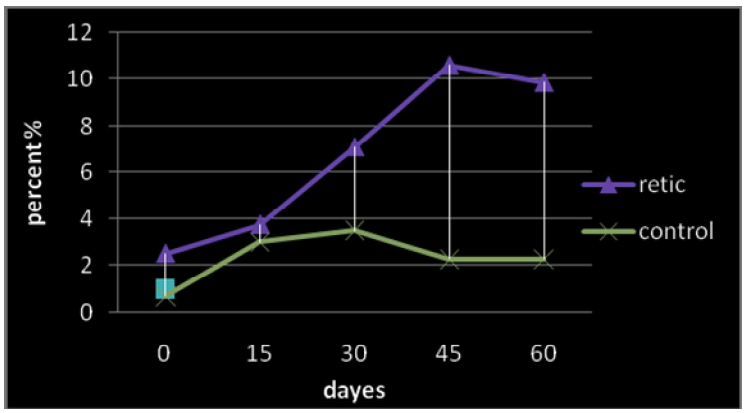

Figure 2 Mean changes in reticulocytes percent in Diclofenac treated group as compared with control group. retic= reticulocytes

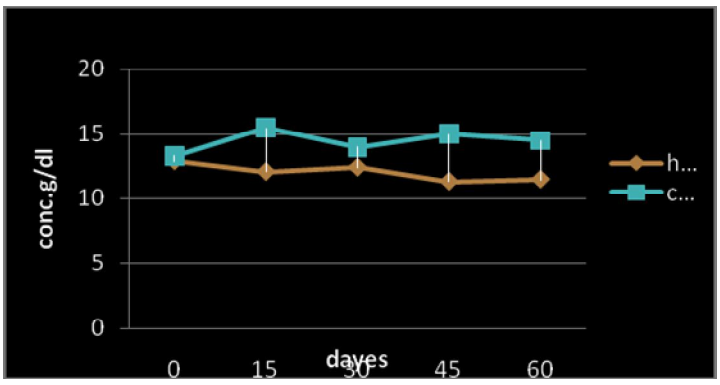

Figure 3 Mean changes in hemoglobin conc. (g/dl) in Diclofenac treated group as compared with control group. Hb=hemoglobin

Effect of Diclofenac sodium (0.3 mg/kg) on WBCs related parameters in Diclofenac treated group compared with control group.

This study reveals non significant difference (p>0.05) in WBC count, monocytes & eosinophiles percentages of Diclofenac treated group as compared with control group. while there was significant decrease (p< 0.05) in neutrophils percent of Diclofenac treated group as compared with control group, Figure (4). Add to that ,there was significant increase (p< 0.05) in lymphocytes percent of Diclofenac treated group as compared with control group in which the highest lymphocytes percent at day 4, Figure (5).

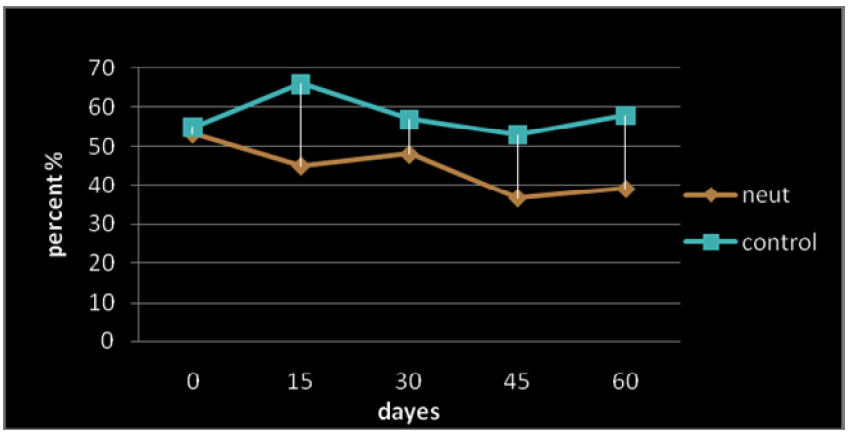

Figure 4 Mean changes of neutrophils percent of Diclofenac treated group as compared with control group. neut=neutrophil

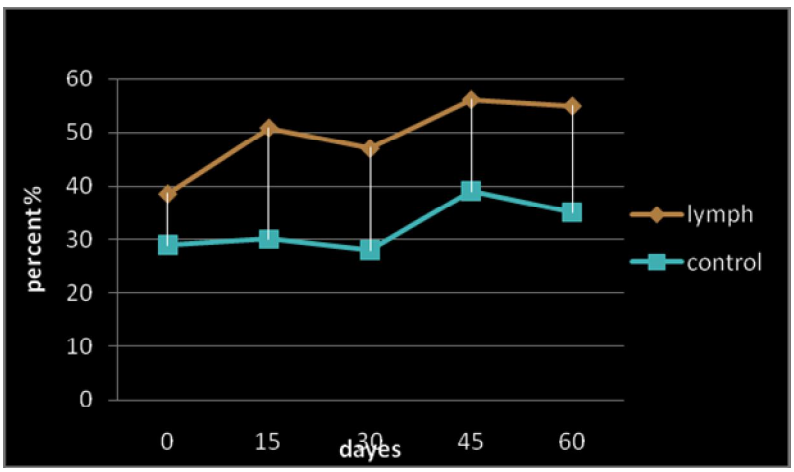

Figure 5 Mean changes of lymphocytes percent of Diclofenac treated group as compared with control group.

Bone marrow

Diclofenac sodium treated animal :Bone marrow smear revealed increase in lymphocytes count & bone marrow erythroid activity such that M:E ratio reversed (1:2)as compared with

control bone marrow smear in which M:Eratio (2:1)Figure(7).

Coomb's test revealed negative results except in: one from Diclofenac treated group and On the other hand Radial immune diffusion assay showed that there was increase in IgG conc. of the same rabbit. While there were no changes observed in IgM conc.of treated group as compared with control group.

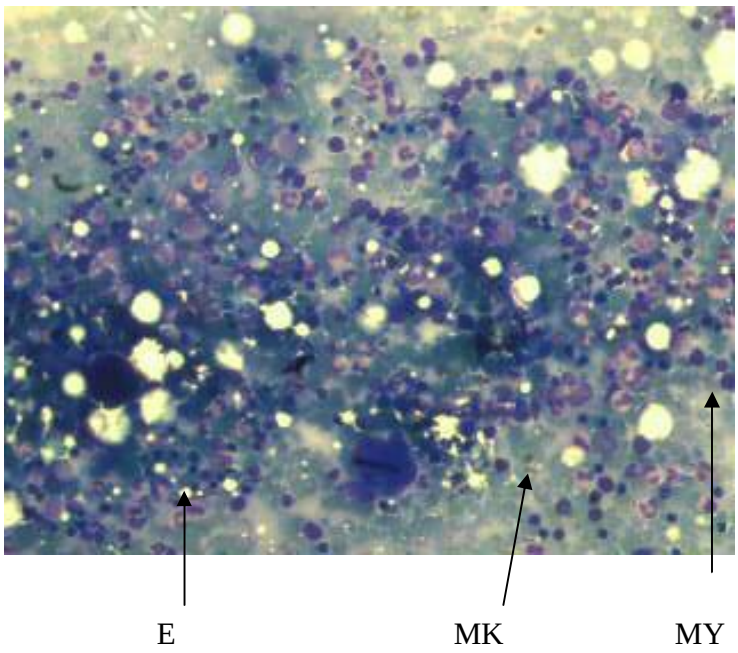

Figure 6 Bone marrow smear: control;normal MY:E ratio MK= Megakariocyte, MY=Myloid, E=Erythroid cells, Magnification power 10x40.

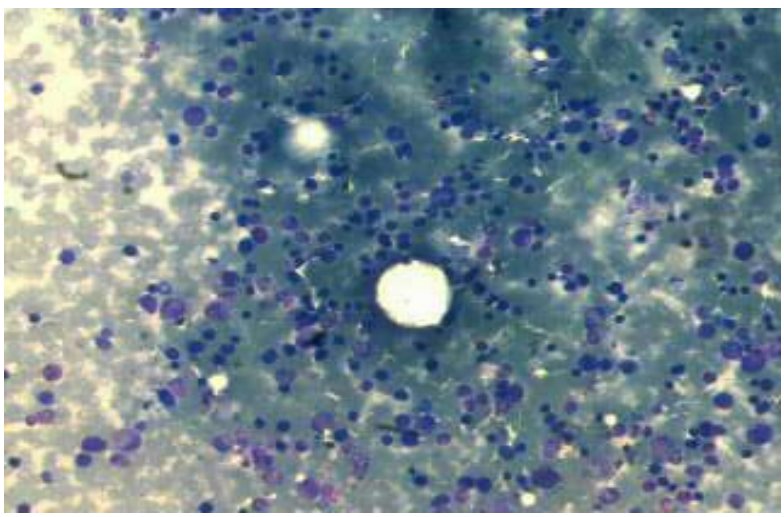

Figure 7 Bone marrow smear, Diclofenac treated group, reversed MY: E ratio. Magnification power 10x40.

Discussion

Blood dyscrasias related to NSAIDs therapy are clinically important. All cell lines can be affected, resulting in red cell aplasia, hemolytic anemia, Agranulocytosis, thrombocytopenia, and aplastic anemia.

Diclofenac sodium treated group: Red cell related parameters There was significant decrease in hematocrit percent and hemoglobin conc. in Diclofenac treated group as compared with control group, Tables (1). Additionally, there was significant increase in reticulocytes percentage as compared with control group, Tables (1). This result agreed with Sachs et al,2004[17] who found patients with Diclofenac induced immune hemolytic anemia produce broad spectrum of anti Diclofenac –RBCs anti bodies. But according to the result of this study, Diclofenac induced anemia was not anti body mediated as coomb's test was negative and radial immune diffusion assay for IgG conc. of Diclofenac treated group was within normal level, except in one of the animals treated with Diclofenac. So, we expect that hemolytic anemia induced by Diclofenac may be due to cellular immunity rather to humoral immunity and this can be proved in part by increase in lymphocytes percent in peripheral blood and bone marrow smear of Diclofenac treated group.According to the association of hemolytic anemia with duration of treatment we found that hemolytic anemia was accompanied with Diclofenac treatment and was drug dependent as upon discontinuation of treatment we noticed correction of hemoglobin conc. about 1g/dl in two weeks Figure (1) and (3). Also, we found that anemia increased with chronicity of Diclofenac treatment.

White blood cell related parameters: there was significant decrease in neutrophiles percents and increase in lymphocytes percents in Diclofenac treated group as compared with control group, Table (2) . This agreed with Van der Klauw *etal*, 1998[23] who found that Diclofenac is one of the drugs associated with probable neutropenia. And Ibanez *etal*, 200524 who observed that Diclofenac sodium is one of drugs that associated with significant risk of neutropenia. Also, agreed with Gutting B.W,2002 [16] who found that there was increase in popletial lymph node size in patients treated with Diclofenac sodium , while these results disagreed with EMEA ,2004[7] who found that there was increase in white blood cells and segmented neutrophils in male Beagle dogs treated with Diclofenac sodium 1mg/kg for 90 days this may be due to species and dose variation. Neutropenia was accompanied with treatment and may be due to Diclofenac induced antibodies against peripheral neutrophils or their bone marrow precursor which was difficult to be identified[25]. On the other hand there were no significant changes in eosinophiles and monocytes percent Table (2) as we did not observe allergic signs and severe infection in study period .**Bone marrow :**There was increase in erythroid precursors with decrease in granulocytes precursors in such a way that the ratio reversed 1:2 and lymphocytosis observed in bone marrow smear of Diclofenac treated animal, Figure (7), and this result confirms peripheral hematological changes . Coombs test

was positive in one of Diclofenac treated animals and radial immune diffusion assay for Diclofenac treated group were within normal level for both IgG and IgM conc. except one of the animals which showed high IgG conc. this could be explain the possibility of humoral immunity role in hematological changes induced by Diclofenac in this study[18].On the bases of the results of this study, it can be concluded that:Diclofenac cause anemia during study period and this anemia is hemolytic anemia. Diclofenac may induce anemia by humoral and/or cellular immune mechanisms.Diclofenac cause no significant change in total WBC count while, they cause significant neutropenia and lymphocytosis along study period possibly due to immune modulatory effects.

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