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HOW LONG-TERM INTAKE OF SODIUM FLUORIDE (NAF) IN DIFFERENT DOSES AND 7,12 DIMETHYLBENZ(A)ANHTRACENE (DMBA) AFFECT THE ERYTHROCYTE PARAMETERS IN RATS?

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ABSTRACT

This study was aimed to search the effect of Sodium Fluoride (NaF) and 7,12 dimethylbenz(a)anthracene (DMBA) to erythrocyte fragility and parameters in rats. The nine groups were formed and each group contained 8 animals. Group 1: Control group (without any treatment). Group 2: Sesame oil (vehicle for DMBA). Group 3: 1 ppm NaF. Group 4: 15 ppm NaF. Group 5: 30 ppm NaF. Group 6: DMBA.Group 7: 1 ppm NaF + DMBA. Group 8: 15 ppm NaF + DMBA. Group 9: 30 ppm NaF + DMBA. Fluoride was added into the animals' drinking water in the form of NaF once a day, for 12 weeks. DMBA (10 mg/kg) was administered once a week and in a total of 12 weeks with oral gavage. Erythrocyte fragility was analyzed with osmotic hemolysis method and erythrocyte parameters with blood cell counter in whole blood. At 0.4% NaCl concentration groups 4, 5, 6, 8, and 9 showed significantly higher erythrocyte fragility values than control group ($p \leq 0.05$). At 0.5% NaCl concentration, groups 6, 8 and 9 showed significant increase in erythrocyte fragility compared to other groups ($p \leq 0.05$). The erythrocyte and hematocrit values were found significantly high in group 5 ($p \leq 0.001$) and group 4 ($p \leq 0.01$) while it was found low in all groups with DMBA (6, 7, 8, 9) ($p \leq 0.05$) compared to control group. Hemoglobin amount in group 5($p \leq 0.01$) and group 4 ($p \leq 0.05$) were significantly higher than other groups. MCV and MCH in group 5 were significantly lower and these values in all groups with DMBA (6, 7, 8, 9) were determined significantly high compared to other groups. RDWC in group 5 ($p \leq 0.001$) and group 4 ($p \leq 0.01$) and in all groups with DMBA (6, 7, 8, 9) ($p \leq 0.05$) was significantly increased compared to other groups. As a result, exposure to high doses of floride and DMBA may cause augmented erythrocyte fragility, abnormal erythrocyte parameters and anemia. Therefore, measures must be taken to protect the health of all living organisms in area exposed to high levels of fluoride and DMBA.

Key words: Erythrocyte fragility, fluoride, DMBA, PAH, anemia.

INTRODUCTION

Fluoride is an important element for bone and teeth structure. However, it is not found in free form in nature and taken into human body via compounds containing fluoride. Lack of fluoride is rarely encountered but excessive intake can be an important health hazard. Acute exposure to fluoride is rare and can be due to intended purposes such as suicide or accidental consumption. Chronic fluoride intoxication can be formed when low doses of inorganic fluorides are consumed in the long time periods. Chronic fluoride intoxication is known as "fluorosis" (Comba, 2013).

Existence of fluoride rich soil and ground water resources due to volcanic nature or via industrial pollution and intake of vegetation growing in such environments may end up with health problems related with fluorosis. Some countries have such regions where people live in areas in the catchment of volcanic mountains. Surrounding of Tendurek Mountain or Ararat Mountain in Turkey are examples for such areas. High exposure to fluoride may be a risk factor for the formation of cancer in human. In addition, they cause other health problems (Kilicalp *et al*., 2004; Mert *et al*., 2016; Comba and Cinar, 2016). According to World Health Organization reports up to 1.5 ppm fluoride is accepted as safe and higher levels are warned for health hazards (WHO, 1994). Oto (2002) reported that water samples from Muradiye region (Turkey, Van province) contained 0.129 to 1.355 ppm and villages around Çaldıran (Turkey, Van province) contained 0.283 to 4.325 ppm fluoride (Oto, 2002). People and animals living in such rural areas are under threat of fluorosis. The adverse effect of fluoride on haematopoietic organs (Eren *et al*., 2005) and suppression of the functions of blood cells have been reported (Curnette, 1979).

Another important health hazard encountered in such rural areas is the unintended exposure to polycyclic aromatic hydrocarbons (PAH) due to baking of bread at tandouri or grilling meat on solid fuel sources containing organic or processed hydrocarbon sources such as petrol. PAH compounds are potent carcinogens (WHO, 1994).

Sources of exposure to PAH compound can vary according to condition of settlement. Urban inhabitants may encounter with such pollutants by exhaust gases from cars and coal gases whereas rural people may be exposed to them by cooking activities (Granberg *et al* 2000, Clemens 1991). 7, 12 dimethylbenz (a) anthracene (DMBA) is among those PAH compounds and known to cause tumor formation in various tissues in both human and animals. Current literature states an oxidative damage and an inhibition of antioxidant enzymes (Ozturk *et al* 2006). Antioxidant defense system is important for protecting tissues against tumor formation. Therefore, suppression of this defence attenuates protective mechanisms against cancer formation.

Metabolizing capacity of PAHs varies among animal tissues. The highest metabolizing capacity in descending order takes place in liver, lung, intestinal mucosa, skin and kidneys. PAH metabolism may also take place in nasal tissues, mammary glands, spleen, brain, hair follicles, erythrocytes, platelets, leukocytes, gonads, placenta and uterus (Anderson *et al*., 1989).

Major cellular elements of blood are formed by erythrocytes which comprises most of the packed cell volume in hematocrit count. Their hemoglobin content gave them both their red color as well as their oxygen carrying function. Because of their high surface to volume ratio they have a high shape deformation property without losing their integrity. Although they are quite strong against mechanical stresses encountered during passage from small capillaries they are vulnerable against osmotic changes. Therefore, blood pH and osmotic value are kept constant within a narrow fluctuation. This vulnerability of erythrocytes gave way to an important laboratory test. This test is known as erythrocyte osmotic fragility test which is an indicator of membrane stability. This stability is affected from different factors such as oxidative stress mediated lipid peroxidation, protein carbonylation, loss of spectrin which is the major mechanical support mesh of erythrocyte cell membrane and etc. At a certain level erythrocytes lose their resistance against such changes and start to lyse.

There are studies conducted on hormones (Cinar and Selcuk, 2005; Comba and Cinar, 2016), electrocardiogram (Kilicalp at al; 2004) under exposure to NaF, however no study was performed on erythrocyte osmotic fragility and parameters under concomitant exposure to fluoride and DMBA in the long term. This study aims to assess erythrocyte osmotic fragility and parameters (erythrocyte count, haemoglobin content, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, distribution frequency of erythrocytes) in wistar albino rats exposed to DMBA, different concentrations of fluoride and concomitant administration of these two chemicals.

MATERIALS AND METHODS

Chemicals: Sodium fluoride (NaF) (Sigma-Aldrich, S7920), 7,12 dimethylbenz(a)anthracene (DMBA) (Sigma, D3254) and sesame oil (Sigma-Aldrich, S3547) were purchased from Sigma. NaF was dissolved in tap water and DMBA was suspended in sesame oil.

Animal material: The seventy-two male Wistar albino rats were used in this study (200-220 gr). Rats were housed in standard plastic cages at Van Yuzuncu Yil University, Animal Experiments Unit. 12 hours' light/12 hours' dark period was kept constant. Room temperature was kept at 22 ± 2°C. Water and standard pellet food was given *ad libitum*. The study was done in accordance with rules of Ethical Committee of Van Yuzuncu Yil University.

Experimental Design: The nine groups were determined and eight rats were selected randomly for each group. Fluoride was added into the animals' drinking water in the form of NaF once a day, for 12 weeks. DMBA (10 mg/kg) and sesame oil (vehicle for DMBA) were given once a week and in a total of 12 weeks with oral gauge. Groups and treatments to groups are as follows:
Group 1: Control group (without any treatment). Group 2: Sesame oil. Group 3: 1 ppm NaF. Group 4: 15 ppm NaF. Group 5: 30 ppm NaF. Group 6: DMBA.Group 7: 1 ppm NaF + DMBA.Group 8: 15 ppm NaF + DMBA. Group 9: 30 ppm NaF + DMBA

Blood Collection: At the end of study, blood samples were collected from all the rats sacrificed under anesthesia with an i.p. injection of 70 mg/kg of ketamine HCl (Ketalar, Pfizer) and 10 mg/kg xylazine HCl (Rompun, Bayer). Blood samples were taken from hearts with sterile injector and placed into tubes with EDTA.

Erythrocyte fragility measurements: Erythrocyte fragility was measured at 546 nm with a spectrophotometric device. Solutions containing different NaCl and distilled water were prepared between 0.1, 0.4, 0.5 and 0.85 %. Buffering of the solutions were assured by adding sufficient amount of Na_2HPO_4 and NaH_2PO_4. Following withdrawal of blood in tubes containing K-EDTA blood samples were incubated for 24 at ambient temperature. 30 microliters of blood were added into dilution tubes. Each tube contains 2 ml solutions. Mixed blood was incubated for 30 minutes and centrifuged for 5 minutes (3000 rpm). Supernatant fractions were placed into the spectrophotometer and recorded.

Erythrocyte Parameters Assay: Red blood cell (RBC) counts, haemoglobin content (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and distribution frequency of red blood cell (RDWC) were determined using rat mode of

veterinary blood cell counter (Abocus Junior Vet-5, Austria) in whole blood.

Statistical Analysis: Data were presented as mean±standard deviation. SPSS version 20 was used for statistical analysis. Kruskal-Wallis and Duncan tests were used for comparison of erythrocyte fragility and parameters.

RESULTS

Erythrocyte fragility: Erythrocyte fragility values were given at Table 1and Figure 1. Erythrocyte fragility results show an increase in fragility at 0.4% NaCl. Erythrocyte fragility percent was found as 67% for control at this concentration. At this concentration similar results were observed for Sesame oil (69%), NaF1ppm (70%), NaF1ppm+DMBA (71%). However, all other groups showed significantly higher erythrocyte fragility values compared to control. At 0.5% NaCl concentration, DMBA, NaF15ppm+DMBA and NaF30ppm+DMBA showed significant increase in erythrocyte fragility. Erythrocyte fragility observed at NaF30ppm+DMBA almost doubled fragility at NaF15ppm+DMBA group.

Table 1. Erythrocyte osmotic fragility values

Grup	0.1	0.4	0.5	0.85
Control	100	67	2	0
Sesame	100	69	5	0
NaF1ppm	100	70	3	0
NaF15ppm	100	**87***	5	0
NaF30ppm	100	**87***	4	0
DMBA	100	**90***	**8***	0
NaF1+DMBA	100	71	2	0
NaF15+DMBA	100	**89***	**15***	0
NaF30+DMBA	100	**91***	**25***	0

n=8 for each groups. Results were presented as mean percent hemolysis at different concentrations of NaCl. * p≤0.05.

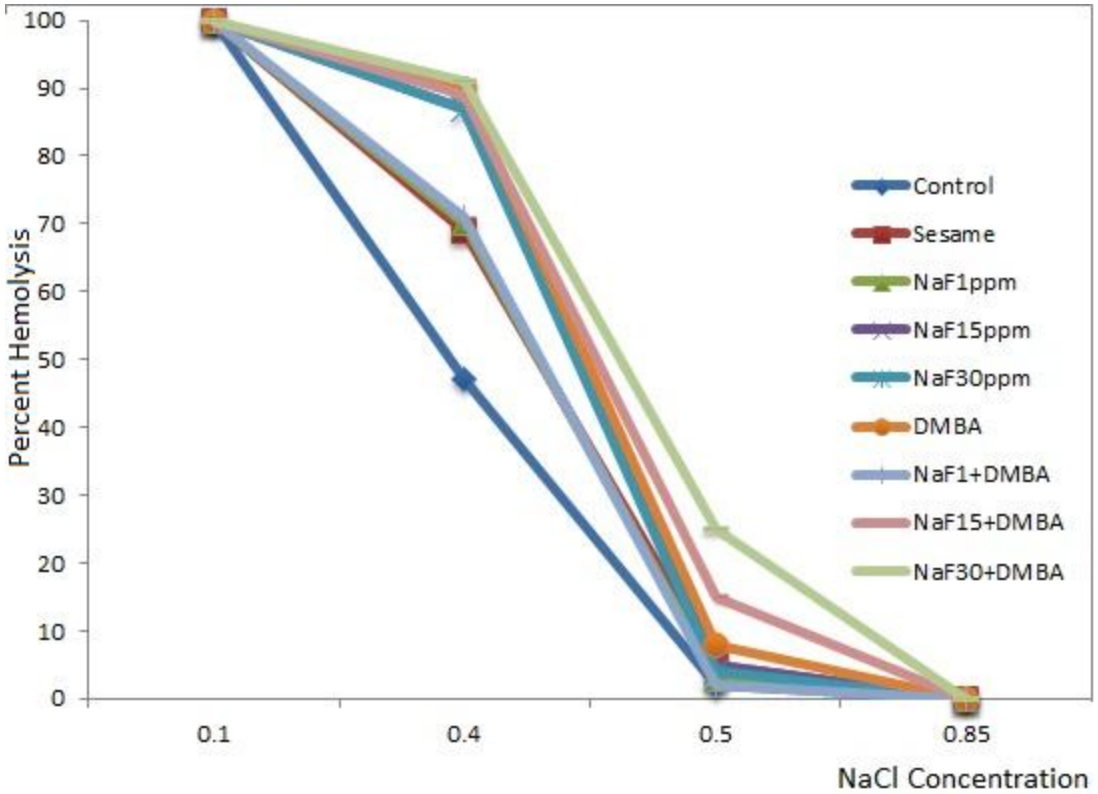

Figure 1. Erythrocyte osmotic fragility results of groups
Results were presented as percent hemolysis at different concentrations of NaCl. *p≤0.05.

Erythrocyte Parameters

Table 2. Results of RBC, HGB, HCT, MCV, MCH, MCHC, RDWC in all groups.

	RBC (10^{12}/l)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDWC (%)
Control	8.43±0.47*	13.77±0.39	47.16±1.90*	56.17±1.04*	16.38±0.90*	29.23±1.11	15.45±0.44
Sesame	8.44±0.60*	13.80±0.64	47.60±2.16*	56.50±1.64*	16.38±0.51*	28.93±0.29	15.13±0.72
NaF1ppm	8.85±0.50*	14.28±0.53	48.59±2.28*	56.00±1.26*	16.48±0.45*	29.40±0.37	15.15±0.51
NaF15ppm	10.18±0.59**	16.31±0.80**	52.36±2.91**	52.88±1.25**	15.69±0.30**	28.84±0.51	17.59±0.41**
NaF30ppm	13.08±1.22***	18.00±1.39***	56.41±3.54***	49.83±1.79***	14.10±0.93***	28.37±1.30	20.05±3.44***
DMBA	7.76±0.63	13.17±0.61	44.02±2.60	57.14±1.21	17.50±0.76	30.60±1.27	16.51±0.86*
NaF1+DMBA	7.97±0.76	13.58±1.03	43.67±3.86	57.40±1.52	17.24±0.72	30.26±1.14	16.92±0.89*
NaF15+DMBA	7.69±1.22	13.27±0.45	43.74±3.18	57.67±1.75	17.52±0.78	28.90±1.53	16.58±0.35*
NaF30+DMBA	7.36±0.23	13.33±0.29	43.25±2.88	57.83±2.23	17.53±0.78	29.85±1.92	16.32±0.58*

RBC, red blood cells; HGB, Hemoglobin; HCT, Hematokrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration, RDWC, distribution frequency of red blood cell.
The results are the means± SD for 8 rats in each group. Significantly different between groups: $^{***}P \leq 0.001$; $^{**}P \leq 0.01$, $^{*}P \leq 0.05$.

The erythrocyte parameters (RBC, HGB, HCT, MCV, MCH, MCHC, RDWC) are shown in *Table 2*. According to the *Table 2* the RBC and HCT values were found significantly high in group 5 ($p \leq 0.001$) and group 4 ($p \leq 0.01$) while was it found low in all groups with DMBA (groups 6, 7, 8, 9) ($p \leq 0.05$) compared to control group. HGB account in group 5 ($p \leq 0.01$) and group 4 ($p \leq 0.05$) were significantly higher than other groups. MCV and MCH in group 5 were significantly low compared to all groups with DMBA (groups 6, 7, 8, 9) were determined significantly high compared to other groups. RDWC in group 5 ($p \leq 0.001$) and group 4 ($p \leq 0.01$) and in all group with DMBA (groups 6, 7, 8, 9) ($p \leq 0.05$) significant increase compared to other groups were obtained with highest value in group 5.

DISCUSSION

This study was conducted to assess any adverse effect of fluoride solutions at different doses, DMBA and their concomitant administration on the erythrocyte fragility and parameters of wistar albino rats.

Fluoride-induced disorders in hematopoietic organs in mice and in human hematopoietic progenitor cells are known (Machalinska *et al*.,2002). Recent studies (Arpita and Bidyut, 2012; Khan *et al*., 2013; Atmaca *et al*., 2014) reported that intensive fluorid cause decreases in the RBC, HGB, HCT, MCV, MCH and MCHC. The decrease in HGB was suggested to be due to toxic effect of fluoride on the serum level of iron and poor retention of iron (Hoogstratten *et al*., 1965). In a study (Kanwal *et al*., 2016), 5 and 10 mg F/L were given in drinking water on gestation days 6–18 in rats and RBC, HB, HCT, MCV, MCH, MCHC values were reported significantly low according to control. In another study (Samantaa *et al*., 2016) 13 mg/kg body weight was given daily for 16 weeks by oral gavage. Hematological study showed NaF induced damages on RBC count showed a significant decrease ($p \leq 0.01$) compared to normal control group. The significant HCT changes might be due to toxic effects of fluoride on the RBC cell membrane and subsequent shrinkage of cell (Maiti and Das, 2004).

In studies investigating the erythrocyte parameters in intense fluoride toxication of different animal species, significant decrease RBC, HGB, HCT in the fluorotic cattle (Samal *et al*., 2016) and calves (Mandal *et al*., 2015) had been determined compared to the healthy ones. Moreover, fluoride induced anemia was also observed in cattles (Dwivedi *et al*, 2000) bovine (Singh and Swarup, 1994) buffaloes (Dwivedi *et al*.2000, Singh and Swarup, 1994), and goats (Radostits *et al*.,2000, Kant *et al*., 2009).

Our results indicate significant increases of RBC, HGB, HCT and RDWC while decreaes in MCV, MCH in 15 and 30 ppm NaF groups according to control. The finding in our study of widespread markedly changes were shown of erythrocyte parameters especially in 15 and 30 ppm NAF and all DMBA groups' rats.

Employees working in the aluminum factory are exposed to fluoride by air and water, causing a decrease in the amount of hemoglobin. Exposure to fluoride can lead to a decrease in erythropoietin stimulation due to thyroid failure and in degradation of the synthesis of vitamin B12, which is required for hemoglobin synthesis due to the self-destruction of the probiotics, and can result in erythropoesis and anemia (Susheela *et al*., 2013). Similar mechanisms may have been involved in the fluoride exposed pregnant dams as indicated by a significant rise in the plasma bilirubin concentration may also be an additional cause of the significant decrease in the RBC, HGB, HCT, MCH, and MCHC parameters in the fluoride exposed dams compared to those in the

control group (Kanwal *et al*., 2016). But in our study 15 and 30 ppm Sodyum fluoride increased RBC counts, HGB concentration, HCT, RDWC. Increase in these parameters and in mentioned groups may be related to the alteration in the size of erythrocytes. In fact, Samantaa *et al*., (2016) have reported effect of chronic fluorosis toxicity in blood cells of albino rats showed different types of abnormal erythrocytes, poikilocytes, and schistocytes.

Fluoride intoxication depresses bone marrow activity in cattle resulting in normocytic and normochromic anemia due to reduced erythropoesis (Swarup and Singh 1989). No significant change in MCV, MCH and MCHC was observed in fluorotic cattle as compared to healthy cattle (Samal *et al*., 2016). Hovewer, in this study 15 and 30 ppm NaF decreased MCV and MCH. This status may be a presentation of microcystic hypochromic anemia, as indicated in the literature (Jagadish *et al*., 1998). Although RBC, HCT and HGB values increased with 15 and 30 ppm NaF in our study, significant increase in erythrocyte fragility in those groups indicate an untoward erythrocyte production which is also supported with increased RDWC.

The reason for the differentiation of erythrocyte parameters in studies on fluoride toxication may be due to the difference in the dose or application periods. Thus the 50% acute lethal dose (ALD50) of sodium fluoride (NaF) via the oral route in rats was found to be 52 mg/kg body weight (Samantaa *et al*., 2016).

Repeated daily administration of PAHs causes immunosuppression (Laiosa *et al*., 2010) and single doses of 7,12-dimethylbenz(a)anthracene (DMBA) suppress the proliferative activity of erythroid progenitors within 6 hours. Mature bone marrow cells are unaffected as shown by minimal gene expression responses to DMBA (N'jai *et al*., 2011, Larsen *et al*., 2016). These shared progenitor suppression responses suggest that stem cell differentiation to the respective lines are blocked by DMBA metabolites (Rondelli, *et al*., .2016)

In mice, oral administration of 65 mg / kg DMBA (single dose) for 3 weeks significantly reduced the levels of all hematological parameters. It was reported that there was a decrease in HGB, HCT, and RBC by 7, 5 and 20 %, respectively (Farombi *et al*., 1997). This reduction in the value of hematological parameters may result from the effect of reactive metabolites produced from the metabolism of these chemicals on the hematopoietic functions of the spleen, which causes bone marrow or blood cells to be destroyed (Smith *et al*., 1974).

In a study, Zingue *et al* (2016), administered DMBA at a dose of 30 mg / kg for 50 days in rats and determined hematological changes. They reported decrease in RBC, HCT, MCV, MCH and also an increase in MCHC without statistical significance.

Rajalingam *et al*., (2008) investigated erytrocyte osmotic fragility levels to determine the membrane integrity modifying effects of DMBA causing skin cancer in Swiss albino rats. They applied DMBA (25 mg / 0.1 ml of acetone) twice weekly for 8 weeks to create skin squamous cell carcinoma and they saw it at the end of the 15th week as 100%. Red blood cell osmotic fragility was assayed by using specific colorimetric methods. And of the study they were reported that erythrocytes from tumor bearing animals were more fragile than those from control animals.

According to these studies, the use of NaF and DMBA at different doses and time periods has been reported that its different effects are on erythrocyte parameters and fragility, but impact of their concomitant use has not been revealed erythrocyte parameters and fragility. Therefore, these effects of NaF and DMBAwere evaluated in this study.

Our results present a significant increase in fragility with the use of fluoride solutions. DMBA seems to aggravate this effect. In addition, values at 0.5% NaCl suggest a dose dependent fashion of increase in erythrocyte fragility since fragility of NaF30ppm+DMBA is significantly higher than NaF15ppm+DMBA. Mode of action of fluoride on erythrocytes needs further assessment. And also results in this study indicate a significant decrease in RBC count and HCT while an increase in MCV, MCH, in all groups with DMBA groups compared to other groups. HGB and RDWC in all groups with DMBA were found lower than other groups. These findings may be a presentation macrocytic hyperchromic anemia.

In summary in the present study, the RBC and HCT values were found significantly high in NaF30ppm ($p \leq 0.001$) and NaF15ppm ($p \leq 0.01$) groups while they were found low in all groups with DMBA ($p \leq 0.05$) compared to control group. HGB account in NaF30ppm ($p \leq 0.01$) and NaF15ppm ($p \leq 0.05$) groups were significantly higher than other groups. MCV and MCH in NaF30ppm groups were significantly low, in all groups with DMBA were significantly high compared to other groups. RDWC in NaF30ppm ($p \leq 0.001$) and NaF15ppm ($p \leq 0.01$) and in all groups with DMBA ($p \leq 0.05$) were obtained significant increase compared to other groups and the highest value was in NaF30ppm group.

As a result, in this study, it was observed that abnormalities in erythrocytosis and microcytic hypochromic anemia when only taken at different doses (15 and 30 ppm) of NaF and taking together at different doses (15 and 30 ppm) of NaF and DMBA (10 mg / kg) erythropenia and macrocytic hyperchromic anemia for 12 weeks of administration period.

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