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PRECLINICAL ANEMIA PANEL STUDIES OF "ROHITAKARISTA" AFTER CHRONIC ADMINISTRATION TO MALE SPRAGUE-DAWLEY RATS

K. N. Roy Niyati, Md. Rakib Hasan*, Swagata Sarker Lopa, Ishtiaque Ahmed Chowdhury, Marjana Khalil, Maksud Hassan Binoy, M Salahuddin Bhuiya and M. S. K. Choudhuri

Department of Pharmacy, Jahangirnagar University, Savar, Dhaka - 1342, Bangladesh

***Corresponding author e-mail:** rakibju38@gmail.com

ABSTRACT

Rohitakarista (RHT) is an Ayurvedic preparation used as a traditional medicine in the South Asian countries for the treatment of liver disorders, splenomegaly and other spleen related problems. The effect of chronic administration of Rohitakarista on the hematological parameters and serum iron profile was studied in this experiment. The acute toxicity test of RHT recorded no death, even at the highest dose of 80 ml/Kg body weight. The animals were divided into two groups. The first group was given RHT preparation at a dose of 40 ml/Kg body weight for 28 days while the second group that served as the control received water for the same period. After 28 days chronic administration of the RHT preparation to the male Sprague-Dawley rats the following hematological changes were noted. There were a statistically significant decrease in total red blood cell (RBC) count ($p=0.03$) and Hemoglobin content of the blood ($p=0.04$) whereas a highly significant decrease ($p=0.01$) in case of Hematocrit level of the blood. The erythrocytic indices such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red cell volume distribution width (RDW) did not change significantly. A significant decrease was noted in case of serum Iron level ($p=0.044$) whereas a statistically significant ($p=0.039$) increase was noticed in serum Ferritin level.

Keywords: Rohitakarista, Hematology, Ferritin, TIBC, Transferrin

INTRODUCTION

Anemia is a public health problem both in Bangladesh and worldwide [1]. It is defined as a "fall of hemoglobin concentration below a statistically defined threshold laying at two standard deviations below the median of a healthy population of the same age, sex, and stages of pregnancy" [2]. Although pregnant women are most frequently affected, it is also ubiquitous in non-pregnant women and other population groups including children [3]. It has been estimated that around 2 billion people in the world are anemic; most of them are found in low-income countries in Asia and Africa [4]. Iron deficiency has been claimed to constitute the major part of the anemia problem. A logical intervention for its prevention and control has therefore been the provision of iron supplementation during pregnancy [5].

Drug induced anemia is also a major problem in low income countries [6]. There are some drugs (such as Streptomycin, Aspirin, and Ceftriaxone etc.) which can cause severe anemia [7-9]. Ayurvedic medicine also recognized as Ayurveda is one of the world's oldest holistic (whole-body) healing systems. It is regarded as a part of complementary and alternative medicine recognized by World Health Organization (WHO), National Institutes of Health (NIH) and others [10]. They have a good safety profile also [11]. But there are reports of heavy metal contamination (such as lead) in ayurvedic preparations resulting in intoxication [12]. The safety profile of these drugs has not been fully investigated. That is why the present study was undertaken to explore the effect of RHT in the anemia profile after chronic administration of it to the male Sprague-Dawley rats.

Rohitakarista is an Ayurvedic preparation used as a traditional medicine in the treatment of splenomegaly in the rural population [13]. An animal model is suitable for predicting what may happen in a small percentage of humans and it is usual to carry out serum chemistry profile investigations in animals in the course of developing any new medical product [14]. Rohitakarista is included (pages 169-170) in the Bangladesh National Formulary of Ayurvedic Medicine 2011 (Approved by the Government of Bangladesh vide Ministry of Health and Family Welfare Memo No. Health-1/Unani-2/89/(Part-1) 116 dated 3-6-1991). Bangladesh National Formulary of Ayurvedic Medicine is compiled by the National Unani and Ayurvedic Formulary Committee and published by the Bangladesh Board of Unani and Ayurvedic Systems of Medicine, 38, Bangabandhu Avenue, Dhaka-1000 under the authority vested in the Board vide section 13(j) of the Bangladesh Unani and Ayurvedic practitioners Ordinance, 1983 in collaboration with the World Health Organization [15]. Permission to manufacture at industrial scale is printed in page no. 535 (column 2: Product code 16.60). Directorate of Drug Administration has issued Notification DA/Admin/1-10/96/6212 dated 19th October 1996 has issued license under Drug Act, 1940 and Rules there under and Drug (Control) Ordinance 1982 for local manufacture and sale in Bangladesh (Published Bangladesh Gazette #24 Part VI dated 11 June, 1998.) At present a good number of Ayurvedic manufacturers are manufacturing and marketing the Classical Ayurvedic Medicinal Preparation [16-22].

MATERIALS AND METHODS

Drugs, Chemicals and Reagents: For the toxicological study, Rohitakarista was collected from Sri Kundeswari Aushadhalaya Limited, Chittagong. Ketamine injection was purchased from ACI Pharmaceuticals Limited, Bangladesh. All other reagents, assay kits and chemicals used in this work were purchased from Human GmbH, Wiesbaden, Germany.

Experimental Animals: Six to eight-week old male Sprague-Dawley rats bred and maintained at the animal house of the Department of Pharmacy, Jahangirnagar University, were used in the toxicological experiment. These animals were apparently healthy and weighed 60-70 g. The animals were housed in a well-ventilated clean experimental animal house under constant environmental and adequate nutritional conditions throughout the period of the experiment. They were fed with rat chow prepared according to the formula developed at Bangladesh Council of Scientific and Industrial Research (BCSIR). Water was provided *ad libitum* and the animals maintained at 12 hours day and 12 hours night cycle. All experiments on rats were carried out in absolute compliance with the ethical guide for care and use of laboratory animals approved by Ethical Review Committee, Faculty of Life Sciences, Department of Pharmacy, Jahangirnagar University.

Experimental Design

Acute toxicity study: The acute oral toxicity test was performed following the guidelines of Organization for Economic Co-operation and Development (OECD) for testing of chemicals with minor modifications (OECD Guideline 425) [23]. Sixteen female mice (non-pregnant, 30-40 g body weight) were divided into four groups of four animals each. Different doses (50 ml/Kg, 60 ml/Kg, 70 ml/Kg and 80 ml/Kg) of experimental drug (RHT) were administered by stomach tube. The dose was divided into two fractions and given within 12 hours. Then all the experimental animals were observed for mortality and clinical toxicity signs (general behavior, respiratory pattern, cardiovascular signs, motor activities, reflexes and changes in skin and fur texture) at 1, 2, 3 and 4 hours and thereafter once a day for the next three days following RHT administration.

Chronic toxicity studies: Prior to the experiment, rats were randomly divided into 2 groups of 8 animals each. One group was treated with RHT and another was used as a control. The control animals were administered with distilled water only as per the same volume as the drug treated group for 28 days. For all the pharmacological studies the drugs were administered per oral route at a dose of 40 ml/Kg body weight [24]. After acclimatization, Ayurvedic medicinal preparation was administered to the rats by intra-gastric syringe between the 10 am to 12 am daily throughout the study period. All experiments on rats were carried out in absolute compliance with the ethical guide for care and use of laboratory animals. The experiment animals were marked carefully on the tail which helped to identify a particular animal. By using identification mark, responses were noted separately for a particular period prior to and after the administration [25].

Blood Samples Collection and Preparation of Serum: At the end of the 28 days treatment period, after 18 hours fasting, rats from each group were anaesthetized by administration (i.p) of ketamine (500 mg/Kg body weight) [26]. Blood samples were collected from post vena cava of rats into EDTA

(Ethylene di-amine tetra acetic acid) sample tubes for hematological analysis and into plain sample tubes for serum generation for biochemical analysis. Serum was obtained after allowing blood to coagulate for 30 minutes and centrifuged at 4000 g for 10 minutes using bench top centrifuge (MSE Minor, England). The supernatant serum samples were collected using dry Pasteur pipette and stored in the refrigerator for further analysis. All analyses were completed within 12 hours of sample collection [27].

Determination of Anemia Profile Studies: Anemia profile studies involved analysis of parameters such as Red Blood Cells (RBCs) level determined by Electrical Impedance method [28], Hemoglobin (HGB) level determined by Modified hemiglobincyanide method [29], Serum Transferrin determined by Turbidity method [30], Total Iron Binding Capacity (TIBC) and the serum ferritin level were also determined [31, 32].

MCV, MCH and MCHC are calculated according to the formula as given by Wintrobe [33] and Diem and Clenter [34]:

MCV = [HCT (%) / RBC count (millions)] X 10

MCH = [Hb (g/dL) / RBC count (millions)] X 10

MCHC = [Hb (g/dL) / HCT (%)] X 100

Transferrin saturation (%) is calculated according to the following formula:

Transferrin saturation (%) = [Serum Iron x 100] / TIBC [35]

Statistical Analysis: The data were analyzed using independent sample *t*-test with the help of SPSS (Statistical Package for Social Science) Statistics 11.5 package (SPSS Inc., Chicago Ill). All values are expressed as mean ± SEM (Standard Error of the Mean) and $p<0.05$, $p<0.01$, $p<0.001$ was taken as the level of significance.

RESULTS

Acute toxicity study: The drug (RHT) administered up to a high dose of 80 ml/Kg produced no mortality of the experimental animals. Thus the LD_{50} (Median Lethal Dose) value was found to be greater than 80 ml/Kg body weight. The animals did not manifest any sign of restlessness, respiratory distress, general irritation or convulsion. Since RHT is in the clinical use for dyspepsia, debility and arthritis treatment for many years, a limit test was performed in acute oral toxicity study. According to the OECD test guideline 425 when there is information in support of low or non-toxicity and immortality nature of the test material, then the limit test at the highest starting dose level (80 ml/Kg body weight) was conducted. There were no mortality and toxicity signs observed at 80 ml/Kg body weight. Therefore, it can be concluded that RHT when administered at single dose is non-toxic and can be used safely in oral formulations.

Chronic Anemia Profile Studies

Effect of RHT on Hematological Profile of male *rats:* In the male rats there was a noticeable decrease in the RBC count, Hemoglobin content and Hematocrit level. There is a statistically significant ($p=0.03$) decrease in the RBC count ($p=0.03$; 13.34 % decrease) and Hemoglobin content ($p=0.04$; 12.94 % decrease) but a statistically highly significant decrease in the Hematocrit level ($p=0.01$; 14.29 % decrease**)** of the blood of the male rat.

The change in the Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC) and Red cell volume distribution width (RDW) were not significant. All the results are presented in Table 2.

Effect of RHT on Serum Iron Profile of male rats: In the male rats, a statistically significant decrease was noted in the Serum Iron level ($p=0.044$; 28.26 % decrease) but increase in the serum Ferritin level was also statistically significant ($p=0.039$; 29.64 % increase). A statistically insignificant ($p=0.144$) decrease was noted in total Iron binding capacity (TIBC) ($p=0.144$; 16.41 % decrease) and Transferrin saturation ($p=0.215$; 10.13 % decrease) in the serum. All the results are presented in Table 3.

DISCUSSION

Effect of RHT on Hematological profile of male rats: Hematological assessment is useful to determine the extent of toxic effects of experimental drug on the blood constituents of an animal. The analysis of blood parameters is closely related to risk evaluation because when tests involve rodents, the hematological system has a higher predictive value of any abnormal toxicity signs and symbols in humans [36]. We found noticeable hemolytic changes on some major hematological parameters. These findings include the possibility of occurrence of anemic condition.

RBCs are vehicles for carrying hemoglobin and function to transport oxygen and remove CO_2. Sufficient oxygen to each cell in the body is the basis of life because oxygen provides the energy for all the normal activities of the body. Only red blood cells are capable of carrying oxygen to cells [37]. Anemia occurs when the numbers of red blood cells (or the Hemoglobin in them) drop below normal and the body gets less oxygen than it needs to function

properly [38]. Anemia can occur from a malfunction at any point in the production, recycling or regulating of red blood cells in the body. In this study, the drug significantly reduces the RBC count and that is why we can say, the drug has the potential to cause anemia.

Measurement of Hemoglobin, the oxygen carrying protein, is a more sensitive and direct test for anemia. Anemia generally is defined as Hemoglobin values below the fifth percentile in a healthy reference population. It is most commonly used to screen iron deficiency. However, Hemoglobin and Hematocrit are late markers of iron deficiency and they are not specific for iron deficiency anemia [39].

In normal conditions, there is a linear relationship between hematocrit and the concentration of hemoglobin. A low hematocrit means a low number of circulating red blood cells and is an indicator of a decrease in the oxygen carrying capacity. A high hematocrit may reflect an absolute increase in the number of erythrocytes or a decrease in plasma volume. In this study, the drug significantly decreases hemoglobin concentration and hematocrit level [40].

The mean corpuscular volume (MCV) is an indicator of iron-deficiency anemia and is known to be decreased when iron-deficiency anemia is present [41]. MCV is useful for categorizing anemia as microcytic, normocytic, and macrocytic. MCV and MCH (the mean corpuscular hemoglobin) values are reduced usually in anemia patients, and the mean corpuscular hemoglobin concentration (MCHC) is reduced in severe disease [39]. The degree of change in red cell indices is associated in part to the duration and in part to the severity of anemia [42]. The red blood cell distribution width (RDW) measures variations in the size of RBCs and increases with iron deficiency [39].

Effect of RHT on Serum Iron profile of male rats:

Ferritin is a storage compound for iron and serum ferritin level is normally associated with total iron stores. As iron stores are reduced, serum ferritin levels decline and it is the earliest marker of iron deficiency. In addition, serum ferritin is an acute-phase reactant that can become elevated in the setting of inflammation, chronic infection or other diseases [39]. In this study, the drug causes a significant increase in the serum ferritin level.

Serum iron concentration can be measured directly and generally decreases as iron stores are depleted. However, serum iron may not reflect iron stores accurately because it is influenced by several additional factors including iron absorption from meals, infection, inflammation and diurnal variation [39]. In this study, the drug causes a significant decrease in the serum iron level.

TIBC quantitatively measures serum transferrin and can be useful in diagnosis of iron deficiency anemia, iron overload and chronic inflammatory disorders [43]. Increased value of TIBC indicates iron deficiency but the normal or even lower value may occur in iron deficiency anemia [44]. Increased levels of TIBC suggest that total iron body stores are low and may be a sign of iron deficiency anemia, polycythemia vera, and may occur during the third trimester of pregnancy [45, 46]. Decreased levels of TIBC may indicate anemia of chronic disease such as hemolytic anemia, hemochromatosis, chronic liver disease, hypo-proteinemia, malnutrition, pernicious anemia and sickle cell anemia [47]. In this study, the drug decreases the TIBC level.

Transferrin saturation (Tfsat) indicates the proportion of occupied iron-binding sites and reflects iron transport rather than storage. Low levels of serum iron also led to the reduction in the percentage of Transferrin saturation [48]. Tfsat decreases before anemia develop, but not nearly enough to identify iron depletion. Tfsat is influenced by the same factors that affect TIBC and serum iron concentration and is less sensitive to changes in iron stores than is serum ferritin [39].

CONCLUSION

From the above experiment it can be concluded that RHT should not be administered chronically at a higher dose as it significantly decreases Red Blood Cell (RBC) count, Hemoglobin, Hematocrit, serum iron but increases serum ferritin level. Further studies should be done by reducing the administered dose. Thus RHT is to be taken only at a dosage of 12–24 ml once or twice a day usually advised after food. If needed, it can be mixed with equal quantity of water.

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Table 1: Name of the ingredients/herbs used in the preparation of "Rohitakarista (RHT)"

Name of Plants / Ingredients	Botanical Name	Used Parts	Family	Amount Used
Rohitaka	*Tecomella undulata*	Stem & Bark	Bignoniacea	4.800 kg
Water for decoction				49.152 L
	reduced to			12.288 L
Guda (Molasses)				9.600 kg
Amalaki	*Embelica officinalis*	Fruit powder	Euphorbiaceae	48 g
Bibhitaka	*Terminalia beleracia*	Fruit powder	Combretaceae	48 g
Cavya	*Pipper retrofractum*	Stem	Piperaceae	48 g
Citraka	*Plumbago zeylanica*	Root	Plumbaginaceae	48 g
Dhataki	*Woodfordia fruticosa*	Flower	Lythraceae	768 g
Ela	*Elettaria cardamomum*	Sod	Scitaminaceae	48 g
Haritaki	*Terminalia chebula*	Fruit powder	Combretaceae	48 g
Pippali	*Pipper longum*	Fruit	Piperaceae	48 g
Pippali mula	*Pipper longum*	Root	Piperaceae	48 g
Rohitaka patra	*Tecomella undulata*	Leaf	Bignoriacea	48 g
Sunthi	*Zingiber officinale*	Rhizome	Zingiberaceae	48 g
Tvak	*Cinnamomum zeylanicum*	Stem & Bark	Lauraceae	48 g

Table 2: Effect of RHT on Hematological profile of rat.

Parameters	Control	RHT	p values	% Change
RBC	7.12 ± 0.08	6.17 ± 0.29	0.03	↓13.34 %
Hemoglobin	12.06 ± 0.48	10.50 ± 0.43	0.04	↓12.94 %
HCT	42.98 ± 0.50	36.84 ±1.51	0.01	↓14.29 %
MCV	60.36 ± 0.39	59.80 ± 0.58	0.44	↑0.93 %
MCH	17.52 ± 0.33	17.36 ± 0.34	0.74	↓0.91 %
MCHC	29.04± 0.60	29.06 ± 0.33	0.97	↑0.07 %
RDW	9.81 ± 0.10	10.82 ± 0.65	0.15	↑10.36 %

Table 3: Effect of RHT on Serum Iron Profile of rat.

Parameters	Control	RHT	p values	% Change
Serum Iron	17.25±1.28	12.38±1.79	0.044	↓28.26 %
TIBC	68.88±6.23	57.57±3.06	0.144	↓16.41 %
Ferritin	4.06±0.44	5.26 ± 0.30	0.039	↑29.64 %
Transferrin saturation (%)	24.32±1.39	21.85±0.93	0.215	↓10.13

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