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Preclinical Anemia Panel Studies of "Makardhvaja" after Chronic Administration to Male Sprague-Dawley Rats

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Abstract

Makardhvaja (MD) is an Ayurvedic preparation used as a traditional medicine in the treatment of sexual dysfunction in the rural population. The effect of chronic administration of Makardhvaja on the hematological parameters and serum iron profile was studied in this experiment. The acute toxicity test of MD recorded no death, even at the highest dose of 80 ml/kg body weight. During the chronic toxicity test, animals were divided into two groups. The first group was given MD preparation at a dose of 40 mg/kg body weight for 28 days while the second group that served as the control received water for the same period. After 28 days of chronic administration of the MD preparation to the male Sprague-Dawley rats, the following hematological changes were noted. Erythrocytic indices such as red blood count (RBC), hemoglobin, Hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell volume distribution width (RDW) did not change significantly. In the male rats, a statistically highly significant ($p = 0.003$) decrease (27.35%) in the serum iron level, an increase (26.42%) in the serum ferritin level, which, though not significant, was prominent ($p = 0.120$), and a statistically very highly significant ($p = 0.001$) decrease (47.05%) in the serum total iron binding capacity (TIBC) were noted.

Keywords: Makardhvaja; Ayurvedic preparation; Hematology; Serum iron level; Ferritin; TIBC

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 lying 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 nonpregnant women and other population groups, including children [3]. It has been estimated that around two 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, Ceftriaxone, etc.) that 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 also have a good safety profile [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 MD in the anemia profile after chronic administration of it to the male Sprague-Dawley rats.

Makaradhwaj is a well-known inorganic preparation of the Ayurvedic Pharmacopoeia used in Ayurvedic antiaging and aphrodisiac treatment [13,14]. Chemically, it is red sulfide of mercury and gold in an uncombined form. It is a sublimed product made from pure mercury, sulfur, and gold.

Eight parts of mercury and one part of gold leaf are mixed together to form an amalgam. To this mixture, 16 parts of sublimed sulfur are added, and the resulting mixture is ground very thoroughly in a stone mortar for 24 h or more until the whole is converted to a lusterless, fine, impalpable powder of uniform consistence. This mixture is then placed in a narrow-mouthed bottle and is gradually heated on a sand bath. On heating, the bottle is filled with reddish fumes of various hues. On cooling, *Makardhwaj* is found deposited in the inner surface of the neck of the bottle.

MD (447 p.) is included in the Bangladesh National Formulary of Ayurvedic Medicine 1992 (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).

Materials and Methods

Drugs, chemicals, and reagents

For the toxicological study, Makardhvaja (MD) was collected from Sri Kundeswari Aushadhalaya Limited, Chittagong. Ketamine injection was purchased from ACI 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 a 12 h day and 12 h night cycle. All

Name of ingredients	Used part	Botanical/English name/Calyx name	Amount used
Shuddha Swarna	Calyx	Purified gold	12 g
Shuddha Parada	Calyx	Herbal purified mercury	96 g
Shuddha Gandhaka	Calyx	Herbal purified sulfur	288 g
Karpasa	Flower	*Gossypium herbaceum*	Quantity sufficient
Kumari	Leaf exudate	*Aloe barbadensis*	Quantity sufficient.

Table 1: Name of the ingredients used in the preparation of MD

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) [15]. Sixteen male mice (30-40 g body weight) were divided into four groups of four animals each. Different doses (50, 60, 70, and 80 ml/kg) of the experimental drug (MD) were administered by a stomach tube. The dose was divided into two fractions and given within 12 h. Then all the experimental animals were observed for mortality and clinical signs of toxicity (general behavior, respiratory pattern, cardiovascular signs, motor activities, reflexes, and changes in skin and fur texture) at 1, 2, 3, and 4 h and thereafter once a day for the next three days following MD administration.

Chronic toxicity studies

Prior to the experiment, rats were randomly divided into 2 groups of 8 animals each. One group was treated with MD 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 mg/kg body weight [16]. After acclimatization, Ayurvedic medicinal preparation was administered to the rats by intragastric syringe between the 10 to 12 am daily throughout the study period. All the experiments on the 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 [17].

Blood sample collection and preparation of serum

At the end of the 28-day treatment period, after 18 h fasting, the rats from each group were anesthetized by the administration (i.p) of ketamine (500 mg/kg body weight) [18]. Blood samples were collected from post vena cava of the 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 min and centrifuged at 4,000 g for 10 min 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 h of sample collection [19].

Determination of anemia profile studies

Anemia profile studies involved the analysis of parameters such as red blood cells (RBC) level determined by the electrical impedance method [20], hemoglobin (HGB) level determined by the modified hemiglobincyanide method [21], serum transferrin determined by the turbidity method [22], total iron binding capacity (TIBC), and the serum ferritin level [23,24]. MCV, MCH, and MCHC are calculated according to the formula given by Wintrobe [25] and Diem and Clenter [26]:

$$MCV = [HCT\ (\%)/RBC\ count\ (millions)] \times 10$$

$$MCH = [Hb\ (g/dL)/RBC\ count\ (millions)] \times 10$$

$$MCHC = [Hb\ (g/dL)/HCT\ (\%)] \times 100$$

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 (MD) administered up to a high dose of 80 ml/kg produced no mortality. Thus, the LD50 value was found to be greater than 80 ml/kg body weight. The animals did not manifest any sign of fever; chronic skin diseases; diabetes; urinary tract disorders; sinuses; nonhealing wounds; fistula; obesity; rheumatoid arthritis; ascites; headache; gynecological disorders; and diseases of ear, nose, throat, and eyes. According to the OECD test guideline 425, when there is information in support of low toxicity or nontoxicity and immortality nature of the test material, 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 MD when administered at single dose is nontoxic and can be used safely in oral formulations.

Chronic anemia profile studies

Effect of MD on hematological profile of male rats

The results of the anemia panel studies are thus: There is a statistically insignificant decrease ($p = 0.681$) [1.69%] in the total number of red blood cells in the male rat. There is a statistically insignificant decrease ($p = 0.641$) [1.91%] in the hemoglobin content in the blood of the male rat. There is a negligible decrease [0.44%] in the hematocrit level of the blood of the male rat, which was statistically not at all significant ($p = 0.904$). There is a statistically insignificant increase ($p = 0.616$) [0.53%] in the mean corpuscular volume, a red cell index of the male rat. There is a negligible decrease [0.14%] in the mean corpuscular hemoglobin, a red cell index of the male rat, which was statistically not at all significant ($p = 0.898$). There is a decrease [0.58%] in the mean corpuscular hemoglobin concentration, a red cell index of the male rat; the decrease, though not significant, was prominent ($p = 0.447$). There is an increase [1.79%] in the red cell volume distribution width, a red cell index of the male rat; the increase, though not significant, was prominent ($p = 0.381$).

Effect of MD on serum iron profile of male rats

In the male rats, a statistically highly significant ($p = 0.003$) decrease (27.35%) in the serum iron level, an increase (26.42%) in

	Control	MD	*p* value	(%) Decrease/ increase
RBC	6.3075 ± 0.20577	6.2012 ± 0.14710	0.681	Decr 1.69%
Hemoglobin	11.0225 ± 0.38264	10.8125 ± 0.21748	0.641	Decr 1.91%
Hematocrit	33.7750 ± 1.02378	33.6250 ± 0.66272	0.904	Decr 0.44%
MCV	53.9625 ± 0.35654	54.2500 ± 0.43219	0.616	Incr 0.53%
MCH	17.4750 ± 0.16448	17.4500 ± 0.09820	0.898	Decr 0.14%
MCHC	32.3875 ± 0.19127	32.2000 ± 0.14392	0.447	Decr 0.58%
RDW	12.5625 ± 0.17107	12.7875 ± 0.18071	0.381	Incr 1.79%

$p^* \leq 0.05$, $p^{**} \leq 0.01$, $p^{***} \leq 0.001$.

Table 2: Effect of MD on hematological profile of rat

	Control	MD	*p* value	(%) Decrease/ increase
Serum iron	27.8750 ±.1.58607	20.2500 ±.1.37256	0.003	Decr 27.35%
TIBC	72.0000 ± 2.63899	38.1250 ± 1.61950	0.001	Decr 47.05%
Ferritin	5.5300 ± 0.33625	6.9912 ± 0.78610	0.12	Incr 26.42%

$p^* \leq 0.05$, $p^{**} \leq 0.01$, $p^{***} \leq 0.001$.

Table 3: Effect of MD on serum iron profile of rat

the serum ferritin level, which, though not significant, was prominent ($p = 0.120$), and a statistically very highly significant ($p = 0.001$) decrease (47.05%) in the serum total iron binding capacity (TIBC) were noted.

Discussion

Effect of MD on hematological profile of male rats

Hematological assessment is useful to determine the extent of the toxic effects of the 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 [27]. We found noticeable hemolytic changes on some major hematological parameters. These findings include the possibility of the 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 [28]. 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 [29]. Anemia can occur from a malfunction at any point in the production, recycling, or regulation of red blood cells in the body. In this study, the drug reduces the RBC count though it is not significant.

The measurement of hemoglobin, the oxygen-carrying protein, is a more sensitive and direct test for anemia. Anemia is generally 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 [30].

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 decreases hemoglobin concentration and hematocrit level [31], but it is not significant.

The mean corpuscular volume (MCV) is an indicator of iron deficiency anemia and is known to decrease when iron deficiency anemia is present [32]. 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 diseases [30]. The degree of change in red cell indices is associated in part to the duration and in part to the severity of anemia [33]. The red blood cell distribution width (RDW) measures variations in the size of RBCs and increases with iron deficiency [30]. If anemia is observed, RDW test results are often used together with MCV results to determine the possible causes of the anemia. It is mainly used to differentiate an anemia of mixed causes from an anemia of a single cause. In our study MCV, MCH, MCHC, and RDW are not at a significant level.

Effect of MD 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. It acts as a buffer against iron deficiency and iron overload [34]. Ferritin is found in most tissues as a cytosolic protein, but small amounts are secreted into the serum, where it functions as an iron carrier. Plasma ferritin is also an indirect marker of the total amount of iron stored in the body; hence, serum ferritin is used as a diagnostic test for iron deficiency anemia [35]. In this study, the drug causes an 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 [30]. In this study, the drug causes a highly significant decrease in the serum iron level.

TIBC quantitatively measures serum transferrin and can be useful in the diagnosis of iron deficiency anemia, iron overload, and chronic inflammatory disorders [36]. The increased value of TIBC indicates iron deficiency, but the normal or even lower value may occur in iron deficiency anemia [37]. 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 [38,39]. Decreased levels of TIBC may indicate anemia of chronic disease such as hemolytic anemia, hemochromatosis, chronic liver disease, hypoproteinemia, malnutrition, pernicious anemia, and sickle cell anemia [40]. In this study, the drug very highly, significantly decreases the TIBC level.

Conclusion

From the above experiment, it can be concluded that MD should not be administered chronically at a higher dose as it significantly decreases serum iron and TIBC level. Further studies should be done by reducing the administered dose to picture out the reason of this discrepancy.

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