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Short Communication

Preclinical Hba1c Level Studies of *Makardhwaj Ras* and *Maha Sankha Bati* after Chronic Administration to Male Sprague-Dawley Rats

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ABSTRACT

Background: *Makardhwaj Ras* (*MRS*) and *Maha Sankha Bati* (*MSB*) are Ayurvedic preparations used as traditional medicines for different clinical indications in the rural population. *MRS* and *MSB* are used in the debility and sprue, respectively. In this study, the effect of these preparations was evaluated on HbA1c levels (percentage) in rats after chronic feeding. The acute toxicological test of *MRS* and *MSB* separately did not record death or signs of toxicity even at the highest dose of 4000 mg/Kg body weight. In the chronic toxicological evaluation, Ayurvedic medicinal preparations *MRS* and *MSB* were administered chronically to the male Sprague-Dawley rats at a dose of 40 mg/kg and 400 mg/kg for *MRS* and *MSB*, respectively, to determine the level of HbA1c (percentage). After 28 days of chronic administration of *MRS* and *MSB*, the level of HbA1c level was determined in the blood and found that both preparation *MRS* and *MSB* increased the level of HbA1c (percentage) in the blood of the rat. *MRS* and *MSB* increased the level of HbA1c in rats, which is a reliable indicator of diabetes mellitus. Therefore, precaution is recommended in the administration of *MRS* and *MSB* for a prolonged period.

Keywords: Ayurvedic preparations, toxicological, HbA1c, administered, blood, diabetes mellitus.

Introduction

Diabetes mellitus is a metabolic disease with multiple etiologies defined by persistent hyperglycemia and disturbances in carbohydrate, lipid, and protein metabolism.[1] Diabetes mellitus is also responsible for abnormalities in insulin production, insulin action, or both.[1] As at 2021, an estimated 537 million people worldwide had diabetes, accounting for 10.5% of the adult population, with type 2 accounting for over 90% of all cases. Diabetes is expected to affect 783 million people, or one in every eight, by 2045, representing a 46% increase from current estimates.[2] The frequency of disease is increasing, most notably in low- and middle-income countries.[3] Diabetes is the leading cause of death worldwide[4,5], with rates similar in men and women. Diabetes-related healthcare costs the world an estimated $760 billion each year.[6] Glycated haemoglobin (HbA1c, glycohemoglobin, glycosylated haemoglobin, haemoglobin, A1C or A1c) is a kind of hemoglobin (Hb) that can be used to diagnose diabetes.[7,8] An ideal level of HbA1c is 48 mmol/mol (6.5%) or below, and diabetes can be diagnosed at 6.5% or above.[7, 8]

MRS and *MSB* are Ayurvedic preparations that are used as traditional medicine in the treatment of rural populations.[9] *MRS* and *MSB* are 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).[9] In this study, HbA1c was measured to evaluate glycemic control in rats after chronic feeding with *MRS* and *MSB*.

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Methods

Drugs, chemicals, and reagents

In this study, finished Ayurvedic medicines *MRS* and *MSB* were purchased from Sri Kundeswari Aushadhalaya Limited, Chittagong, Bangladesh in 2020 (Ingredients list in Table 1, 2).

Experimental animals

Eight-week-old male Sprague-Dawley rats bred and maintained in the animal house of the Department of Pharmacy, Jahangirnagar University, were used in this toxicological experiment. These animals were healthy and weighed 50-70 g. The animals were housed in a well ventilated clean experimental animal house under constant environmental and adequate nutritional conditions throughout the experiment. They were fed with rat chow diet prepared according to the formula developed by the Bangladesh Council of Scientific and Industrial Research (BCSIR). Water was provided ab libitum and the animals were kept on a 12-hour day/12-hour night cycle. The animals were handled according to the ethical guidelines and the study protocol was approved by the Biosafety, Biosecurity, and Ethics Committee of Jahangirnagar University (Ethics approval no: BBEC, JU/ M 2018 (5)2), Savar, Dhaka, Bangladesh.

Experimental Design

Acute toxicity study

The acute oral toxicological test was performed following the guidelines of the Organization for Economic Cooperation and Development (OECD) for the testing of chemicals with minor modifications (OECD Guideline 425).[10] Sixteen male mice (50-70 g body weight) were divided into four groups of four animals each for each drug. Different doses (1000 mg/kg, 2000 mg/kg, 3000 mg/kg, and 4000 mg/kg) of experimental drugs were administered by stomach tube. The dose was divided into two fractions and administered within 12 hours. 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 hours and then once a day for the next three days following administration.

Chronic toxicity study

Before the experiment, rats were randomly divided into 4 groups of 8 animals for each drug. One group was treated with drugs and another as a control. Control animals were administered with distilled water only as per the same volume as the drug-treated group for 28 days. In this study, *MRS* and *MSB* were administered per oral route at a dose of 40 mg/kg and 400 mg/kg, respectively. Following acclimatization, an ayurvedic medicinal mixture was supplied to the rats via an intra-gastric syringe between 10 am and 12 am daily during the study period. All rat tests were conducted strictly with the ethical guidelines for the care and use of laboratory animals. The tails of the experiment animals were neatly marked, allowing them to be identified. By using an identification mark, the responses were noted separately for a particular period before and after administration.

Blood sample collection and serum preparation

At the end of the 28-day treatment period, after 18 hours of fasting, rats from each group were anaesthetized by administration (i.p) of ketamine (500 mg/Kg body weight). Blood samples were collected from the post vena cava of rats into plain sample tubes for biochemical analysis. All analyses were completed within 12 hours of sample collection.[11]

HbA1c assay

HbA1c assay consists of two separate concentration measurements, glycated haemoglobin (HbA1c) and total haemoglobin (THb). The two concentrations were used to determine the percentage of HbA1c or haemoglobin fraction. Individual concentration values of HbA1c and THb generated by the assay were used for calculating the percentage of HbA1c. The whole blood sample was first pretreated with the MULTIGENT haemoglobin denaturant. The erythrocytes were lysed and the haemoglobin was degraded by the proteolytic enzyme, pepsin, to form a hemolysate. Both the THb and the HbA1c concentrations were determined from the same hemolysate. The concentration of total haemoglobin was determined calorimetrically and was based on the method described.[12] The concentration of HbA1c was measured immune-turbidimetrically using a microparticle agglutination inhibition method.[13] ARCHITECT Systems and the AEROSET System automatically measured the individual concentrations of THb and HbA1c in g/dL or mmol/L.

Statistical analysis

The group data are expressed as Mean±SEM (Standard Error of the mean). Independent sample 't' tests were performed for statistical significance analysis. SPSS (Statistical Package for Social Science) for WINDOWS (ver. 25.0) was applied for the analysis of data. Differences between groups were considered significant at $p< 0.05$*, 0.01** and 0.001***.

Table 1: Name of the ingredients/herbs used in the preparation of *MRS*

Sl no	Ingredient	Plant part	Botanical name	Family	Quantity
1	Rasa Sindura	Calcined	Hydragentum (mercury)	Mineral	10 g.
2	Svarna bhasma	Calcined	Processed Gold	Mineral	10 g.
3	Lauha bhasma	Calcined	Purified Iron oxide Fe_3O_4	Mineral	10 g.
4	Lavanga	Flower	*Syzygium aromaticum*	Myrtaceae	10 g.
5	Camphor	Whole plant	*Cinnamomum camphora*	Lauraceae	10 g.
6	Jatiphala	Seed	*Myristica fragrans*	Myristicaceae	10 g.
7	Mrgamada (kasturi)	Musk	*Moschus moschiferus*	Animal Family Moschidae	10 g.
8	Pan	Leaf	*Piper betle*	Piperaceae	Q.S. (for bhavana)

Table 2: Name of the ingredients/herbs used in the preparation of *MSB*

Sl no	Ingredient	Plant part	Botanical name	Family	Quantity
1	Sauvarchala lavana	Calcined	Salt	Mineral	10 g
2	Saindhava lavana	Calcined	Rock salt	Mineral	10 g
3	Vida lavana	Calcined	Black salt	Mineral	10 g
4	Samudra lavana	Calcined	Common salt	Mineral	10 g
5	Romaka lavana	Calcined	Sambhar salt	Mineral	10 g
6	Hingu	Asafoetida	*Ferula asafoetida*	Apiaceae	10 g
7	Shankha bhasma	Calcined	Bhasma of conch shell	Mineral	10 g
8	Chincha kshara	Ash of tamarind	*Tamarindus indica*	Legumes	10 g
9	Shundi	Ginger root	*Zingiber officinale*	Zingiberaceae	10 g
10	Maricha	Fruits	*Piper nigrum*	Piperaceae	10 g
11	Pippali	Root	*Piper longum*	Piperaceae	10 g
12	Gandhaka	Calcined	Purified sulphur	Mineral	10 g
13	Rasa	Calcined	Purified mercury	Mineral	10 g
14	Vatsanabha	Root	*Aconitum ferox*	Ranunculaceae	10 g

The finished product was purchased from Sri Kundeswari Aushadhalaya Limited, Chittagong, Bangladesh

Results and Discussion

For the acute toxicological study, the drugs *MRS* and *MSB* administered separately up to a high dose of 4000 mg/kg produced no mortality (Table 3). Thus, the value of LD_{50} (Median Lethal Dose) was found to be greater than 4000 mg/Kg body weight. The animals showed no signs of respiratory distress, restlessness, general discomfort, or convulsions. Because medications have been used in clinical practice for many years to treat disorders, an acute oral toxicity included a limit test. According to the OECD test guideline 425, when there is information to support the low or non-toxicity and immortality nature of the test material, the limit test at the highest starting dose level (4000 mg/Kg body weight) was performed. There were no mortality and toxicity signs were observed at 4000 mg/Kg body weight. Therefore, it can be concluded that *MRS* and *MSB*, when administered at a single dose are non-toxic and can be used safely in oral formulations. As a result, this served as the basis for dose selection in chronic toxicity research.

Safety profile studies of drugs or plant extracts allow us to determine the pharmacological effects on various organs and biochemical parameters after chronic drug administration.[14-17] In this study, the effect of *MRS* and *MSB* on glucose homeostasis was evaluated after chronic administration. To determine the average plasma glucose concentration over a prolonged period, Ayurvedic medicinal preparations *MRS* and *MSB* were administered chronically to the male Sprague-Dawley rats at a dose of 40 mg/kg and 400 mg/kg, respectively. After 28 days of chronic administration of *MRS* and *MSB*, the following changes were observed. In this experiment, the HbA1c level was determined (Table 4). There was an increase in the HbA1c level [7.93 %] of the male rat after feeding *MRS* drug, the increase although not significant, was prominent (p=0.336). There was an increase [8.43 %] in the HbA1c level of the male rat after feeding *MSB* drug, the increase though not significant, was prominent (p=0.308).

Blood sugar levels can fluctuate from day to day depending on dietary consumption and level of exercise. The haemoglobin A1c test measures glucose management that represents a percentage of the average blood sugar level over the previous 90 days.[18,19] Haemoglobin is present exclusively in red blood cells. [19] Since red blood cells have a three-month lifespan, the HbA1c test will represent the red blood cells that are present in the bloodstream at the time of the test. The higher the glucose level in the plasma, the higher the glucose level inside the red blood cell.[20-24] When assessing the level of the glycated haemoglobin level in the blood, hyperglycemia and diabetes mellitus can be diagnosed. HbA1c is an essential tool to monitor other possibilities, such as cardiovascular disease, immunology, and dyslipidemia.[25-26] An HbA1c of 6.5% is recommended as the cut-off point for diagnosing diabetes.[27] A value of less than 6.5% does not exclude diabetes diagnosed using glucose tests. People with pre-diabetes who have HbA1c levels between 5.7% and 6.4% are at an increased risk of developing diabetes mellitus.[28] Intensive glycaemic control and lower HbA1c levels are followed by a reduction in diabetic sequelae; at HbA1c 7%, there is a 76% reduction in the incidence of diabetic retinopathy, a 35% reduction in the risk of cardiovascular disease, a 54% reduction in diabetic nephropathy, and a 60% reduction in peripheral neuropathy.[28]

This is significant for patients with diabetes, since the higher the HbA1c, the greater the chance of acquiring diabetes-related complications. We discovered varying levels of HbA1c after treating the laboratory animals with the formulations in this investigation. The administration of *MRS* and *MSB* increased the HbA1c level of male rat blood, which was above the normal range, implying that rats diagnosed with diabetes mellitus may develop other complications such as heart disease, chronic kidney disease, and nerve damage in rats.[28]

The public has a frequent misunderstanding that Ayurvedic drugs are safe and free of side effects. Over-the-counter (OTC) sales account for more than 70 % of total Ayurvedic drug sales, resulting in the use of Ayurvedic medicines without the prescription, advice, and monitoring of Ayurvedic physicians. Ayurvedic medicine contains heavy metals such as mercury, lead, and arsenic which impurity may affect the normal function of the liver, brain, and kidney.[29-33] Both *MRS* and *MSB* contain purified mercury in their formulations. Previously, we reported that *MRS* administration causes abnormality in the lipid profile which affects negatively cardiac atherogenic indices, a parameter to predict metabolic syndrome, hypertension, and diabetes mellitus.[34, 35] In our earlier study, we found that Ayurvedic medicines such as *Brihat Khadir Batika* and *Chandraprabha Batika* increased HbA1c in rats due to unknown reasons.[36] In this study, we also found that *MRS* and *MSB* elevated HbA1c levels in rats, however, the specific reason for this abnormality is unknown. More studies are needed for a better understanding of the nature and characteristics of the preparations.

Conclusion

The investigation suggests that *MRS* and *MSB* should not be given in larger doses regularly because they may raise HbA1c levels. Further studies should be conducted by lowering the supplied dose to optimize the ideal dose of *MRS* and *MSB*.

Conflict of Interest

The authors declare no conflict of interest.

Table 3: Acute oral toxicity study of *MRS* and *MSB* in *Swiss* mice

	1000 mg/kg		**2000 mg/kg**		**3000 mg/kg**		**4000 mg/kg**	
	A	**D**	**A**	**D**	**A**	**D**	**A**	**D**
MRS	4	0	4	0	4	0	4	0
% mortality	0		0		0		0	
MSB	4	0	4	0	4	0	4	0
% mortality	0		0		0		0	

Here, n = 4; A = Alive; D = Death

Table 4: HbA1c (percentage) value of control, *MRS* and *MSB*

Parameters	**Control (Mean ± SEM)**	***MRS* (Mean ± SEM)**	**Control (Mean ± SEM)**	***MSB* (Mean ± SEM)**
HbA1c (%)	2.05 ± 0.07	2.21 ± 0.15	2.08 ± 0.15	2.21 ± 0.15
p-value		0.34		0.31
Overall output (%)		Increase 7.93		Increase 8.43

Independent sample 't' tests were done for statistical significance analysis. Values are expressed as Mean ± SEM, n = 8.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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