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SAFETY ASSESSMENT OF A PROTEIN SUPPLEMENT FOR WOMAN (NRL20195PNW) FOR ACUTE AND 28 DAYS REPEAT DOSE TOXICITY STUDIES AS PER OECD GUIDELINES.

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ABSTRACT

Protein supplements are most popular healthcare supplement for overall health. This study evaluated safety profile of the NRL20195PNW a protein supplement specially designed for women with micronutrient fortification for promoting health and well-being. The study is performed as per OECD guidelines for acute and 28 days repeat dose studies in Mice model. The acute toxicity test revealed no observable signs of toxicity or morbidity at 5000 mg/kg. After and during 28 days of repeated dosing of NRL20195PNW, there was no mortality and gross changes in body weight in mice. There were no significant variations in the hematological, biochemical, clinical, histopathological parameters of both NRL20195PNW treated and control group mice during 28 days study. Based on the findings of this study, the no observed adverse effect level (NOAEL) of NRL20195PNW in mice, following oral administration for 28 days was found to be 1000 mg/kg body weight.

INTRODUCTION

A woman's nutritional and fitness presents difference than men as there are indeed some special requirements of women to lose fat, gain muscle, and looks lean. Immune system functionality can be improvised by proper protein supplements. [1] Proteins help hair and nails looking healthy and strong thus improving wellness quotient. Women appear with gender specific nutritional requirements affecting wellbeing; there is great need to address the same. [2] Anatomy and physiological stages of females and role of sex hormones throughout lifetime present nutritional challenges requiring Special attention. Physical demands on females during fertile age affect nutrition, appetite, and health status. This state has short and long term reparations on woman health· [3] Cultural and social factors resulting in stressing gender-specific roles, body shape, and weight put woman on risk of eating disorders, and seeking unhealthy methods to lose weight. Neural and hormonal factors associate to mood swings and perception to stress in woman life· [4]. to address the need, there are protein supplements in market. But to assess the safety profile of such products there is requirement of proper

toxicological studies.Acute toxicity studies are designed to measure the short-term adverse effects of a drug when administered in a single or multiple doses during a period not exceeding 24 h. Results of acute toxicity studies provide:An appropriate dose for multiple dose studies, potential target organs of toxicity if any, an estimation of safe acute doses for humans. [5,6] During the 28 days repeat dose toxicity study, data about observed effects, mortality, body weight, food/water consumption, physical examinations, hematology, blood chemistry, urinalysis, organ weights, gross pathology, and histopathology are collected and analyzed. [7] While protein supplements are used extensively in day to day life, to our knowledge, its toxicity has been not studied rigorously. In this study we have carried out a thorough safety assessment of the NRL20195PNW through the acute and sub-28 days repeat dose toxicity in mice.

MATERIALS AND METHODS

Test material

NRL20195PNW is a protein supplement with micro fortification in a powdered form and intended for human use to promote health and wellbeing. The key ingredients of the formulation are milk solids, protein blend (whey protein concentrate, soy protein isolate, pea protein isolate, rice protein isolate), DHA (omega 3-algal source), Curcumin extract, Shatavari extract, minerals (calcium phosphate tribasic, potassium citrate, sodium citrate, magnesium sulphate, ferric diphosphate, zinc sulphate, manganese sulphate, copper sulphate, sodium selenite), vitamins (ascorbic acid, vitamin e, nicotinamide, calcium-d-pantothenate, pyridoxine hydrochloride, thiamine mononitrate, riboflavin, retinyl acetate, folic acid, d-biotin, phylloquinone, vitamin d2 ergocalciferol, cyanocobalamin).

Acute Toxicity Study in Mice

The study was conducted in compliance with the OECD Guidelines for Testing of Chemicals (No. 423, Section 4: Health Effects) on conduct of "Acute Oral Toxicity - Acute Toxic Class Method" (Adopted: 17th December 2001).

28 days repeat dose toxicity study in Mice.

The study was conducted as per OECD guideline no. 407, adopted on 3 October 2008.

Husbandry:

Following conditions were maintained during husbandry of rodents.

Dose Preparation:

The test article NRL20195PNW was suspended in Distilled water to obtained desired concentration mg/ml just before dosing every day.

Dose Levels & Justification for acute toxicity study

A starting dose level of 2000 mg/kg body weight was selected from one of the four fixed levels (5, 50, 300 and 2000 mg/kg) based on the available historical toxicity data of the ingredients of NRL20195PNW. Food was withheld for overnight prior to administration of the test article. However, mice had an access to water ad libitum. The toxicity of the test article following oral gavage was assessed by stepwise manner. Three female mice were used per step. The mice were observed for incidence of mortality after 24 hrs. & signs of intoxication for 14 days after the administration of test article. Further, the test was repeated on three more female mice at the same dose level and observed for 14 days. [8-10]

Selection of Dose Levels for 28 days repeat dose toxicity study

Dose levels selected for this study were based upon the results and findings of dose range finding study. The highest dose chosen for the study was 1000 mg/kg body weight in mice. As per OECD – 407, generally, at least three test groups and a control group should be used, but if from assessment of other data, no toxic effects would be expected at a dose of 1000 mg/kg bw/d, a limit test may be performed and a dose of NRL20195PNW being 1000 mg/kg bw/d could be used to assess safety and toxicity profile for 28 days.

Study Design for 28 days repeat dose toxicity study

Groups of Five male and Five female mice were administered NRL20195PNW by oral gavage daily at the doses of 1000 mg/kg body weight for 28 days and were sacrificed on day 28 to evaluate its toxicity. Concurrent control group receiving the vehicle was also maintained. Additionally, satellite groups of

five mice per sex receiving test article at 1000 mg/kg level were further observed for a period of 14 days after 28 days for assessment of reversibility, persistence or delayed occurrence of toxicity.

Administration of Test Article

The animals were dosed by oral gavage at approximately the same time each day where possible, using a graduated syringe and a stainless steel intubation needle (16G). The dosage volume administered to individual mice was adjusted according to its most recently recorded body weight. The control group mice received the vehicle (Distilled water) only, by oral gavage at the same dosage volume of 10ml/kg body weight. Treatment in this manner was continued once a day, seven days a week, for a total period of 28 days.

The observations like Mortality, Clinical Signs and examinations, Body weights and Food Consumption were made during the course of treatment period i.e. 28 days. Mice belonging to the satellite group were further observed for a post-treatment period of 14 days to permit evaluation of the persistence or reversibility of any toxic effects, or the occurrence of delayed toxicity.

Clinical pathology

Blood samples were withdrawn on the day of termination of the study, under carbon dioxide anesthesia, from the retro-orbital plexus of all the male and female mice of the test and control group. The samples were collected in tubes containing Heparin (for clinical chemistry) and K-EDTA (for hematology) as an anticoagulant. Food was removed overnight from animals to be sampled for laboratory investigations. Blood smears were also made on glass slides. Clinical chemistry samples were centrifuged at a speed of 3000 rpm for 5 minutes.

Hematology, urinalysis and clinical chemistry

The Hematology estimations and serum chemistry parameters, with their units of measurement were analyzed and provided in observations. Urinalysis was performed on all animals. Urine samples were collected using a battery of specially designed stainless steel urine collection cages. Each Mouse was housed in this cage. Urine samples were collected over a period of 3 hours. Food and water was not offered during this period.

Necropsy examination

On completion of 28 days of treatment, all surviving mice were sacrificed by exsanguinations under carbon dioxide anesthesia and were subjected to complete necropsy. The necropsy performed at termination of the treatment was staggered and conducted on days 28. All the tissues as per OECD 407 guidelines, from all animals, were preserved, in 10% neutral buffered formalin. In addition, samples of any macroscopically abnormal tissues were routinely preserved, along with samples of adjacent tissue where appropriate.

Organ weights

Following organs, from all animals killed at the scheduled sacrifices, were dissected free of fat and weighed wet as soon as possible to avoid drying.

Statistical Analysis

The data evaluated by One-Way ANOVA (Dunett's test) and Student's t test using graph pad prism at the level of significance from the control mean ranges at $p < 0.05$. [11-13]

RESULT AND DISCUSSION:

Acute Toxicity Study in Mice

Survival and clinical signs

There was no incidence of treatment related mortality among the female mice exposed to the test product at and up to the dose of 2000 mg/kg body weight. Treatment of mice with test product and up to the dose of 2000 mg/kg did not induce any remarkable abnormal clinical signs.

Body weights

Test product did not induce any remarkable and significant alteration in the group mean body weights of the female mice treated at and up to the dose of 2000 mg/kg.

Food consumption

The values of average daily food consumption by female mice exposed to test product and up to the dose of 2000 mg/kg were found to be normal.

Ophthalmoscopy

No abnormal findings in cornea, the conjunctivae, the sclera, the iris and fundus were observed.

Necropsy: All animals were subjected to gross necropsy. It is observed that there is no gross

pathological findings were encountered in any of the organs. [8-10]

28 days repeat dose Toxicity Study in Mice

Mortality

There was no incidence of mice exposed mortality among the male and female to the test article (at dose of 1000 mg/kg body wt.) and control group

Clinical Signs: Treatment of mice with NRL20195PNW at the dose of 1000 mg/kg and concurrent control group did not induce any remarkable abnormal clinical signs in either sex. All these signs were noted only during treatment period. In view of comparable incidence of this finding in these groups, it was considered as incidental in nature.

Body Weights: NRL20195PNW did not induce any remarkable and significant alteration in the body weights of the male and female mice treated at the dose of 1000 mg/kg body weight. Control group alsodid not induce any remarkable and significant alteration in the body weights of the male and female mice treated at the dose of 1000 mg/kg body weight (Table 3).

Food Consumption

The value of average daily food consumption by male and female mice exposed to NRL20195PNW at the dose of 1000 mg/kg body weight was found to be comparable to those of the control group. The average daily food consumption per rat per day, computed over the period of 28 days. NRL20195PNW at 1000 mg/kg satellite group was comparable with that of control mice. Similarly, the average daily food consumption by female mice receiving the test article at above dose levels was comparable to that of control mice. After cessation of treatment, during the recovery period too, the values of daily food intake by the intermediate dose group mice were found to be gradually increasing as during the treatment.

Organ Weights:

The groups mean values of absolute and relative weights of kidneys; liver, adrenals, testes/ovaries, spleen, brain, epididymis/uterus, thymus and hearts of male and female mice treated at 1000 mg/kg were found to be comparable with control group miceat termination of the treatment.

Hematology

The group mean values of Haematological parameters viz. hemoglobin (Hb), hematocrit (PCV) and total erythrocyte counts (RBC), total leucocyte count (WBC), differential leucocyte counts (DLC), platelet count, of male and female mice, exposed to NRL20195PNW (at the level of 1000 mg/kg)and control group did not reveal any treatment effect during and at termination of the study (Table 4).

Biochemistry

Treatment of male and female miceNRL20195PNW at the dose of 1000 mg/kg did not exhibit any remarkable treatment effect, on their serum biochemistry parameters. As far as the Haematological and biochemical parameters are concerned, no index of significant alteration in relation to control appeared, after 28 days of treatment in both male and female mice at 1000 mg/kg body weight groups. All values found for these parameters were comparable with the existing control groups (Table 5). [11-13]

Urinalysis: Treatment of male and female mice with NRL20195PNW (at the dose of 1000 mg/kg) and control group did not exhibit any remarkable treatment effect on their urinalysis parameters. The qualitative parameters of urine evaluated for all mice in this study and the microscopic examination of their urine sediment did not reveal any remarkable and treatment related alterations.

Necropsy :NRL20195PNW did not induce any remarkable and treatment related gross pathological alterations in any of the organs / tissues of treated mice at the level of 1000 mg/kg and that of satellite group (1000mg/kg body weight).

Histopathology

All the microscopic changes noticed in this study appeared to be incidental, as their frequency and severity remained identical for the control and the treated animals at 1000 mg/kg. NNRL20195PNW at the dose level of 1000 mg/kg body weight did not produce any histopathological effects in mice under the said experimental conditions (Figure 1).[14-16]

Table 1: Condition maintenance required during Toxicity study

SN	Parameters	Maintenance
1	Environmental conditions	Air conditioned rooms with 10 - 15 air changes per hour, temperature between 21±2 ^{0}C, relative humidity 55 ±5 % and illumination cycle set to 12 hours artificial fluorescent light and 12 hours dark.
2	Accommodation	Individually housed in polypropylene cages with stainless steel grill top, facilities for food and water bottle, and bedding of clean paddy husk.
3	Diet	Pelleted standard rat and mice feed was provided ad libitum
4	Water	Potable water passed through reverse osmosis filtration system and exposed to U.V. ray was provided ad libitum in glass bottles with stainless steel sipper tubes.

Table 2: Details of animals

1	Species	MICE. Rationale - The guideline followed for this test has preferred mice among the species of rodents.
2	Strain	Swiss albino. The strain was selected due to its availability in requisite numbers, and also the availability of historical background data on its characteristics.
3	Number of animals per dose group	Five males and Five females. Additional satellite groups of Five mice of both sex to be used for assessment of reversibility / delayed toxicity.

Table 3: Average of body weight for test and control group

SN	Test	Avg % Gain
1	Female	16.80
2	Male	17.81
SN	**Control**	**Avg % Gain**
1	Female	9.68
2	Male	9.03

Table 3: Average of Animal Hematology Parameters for test and control group

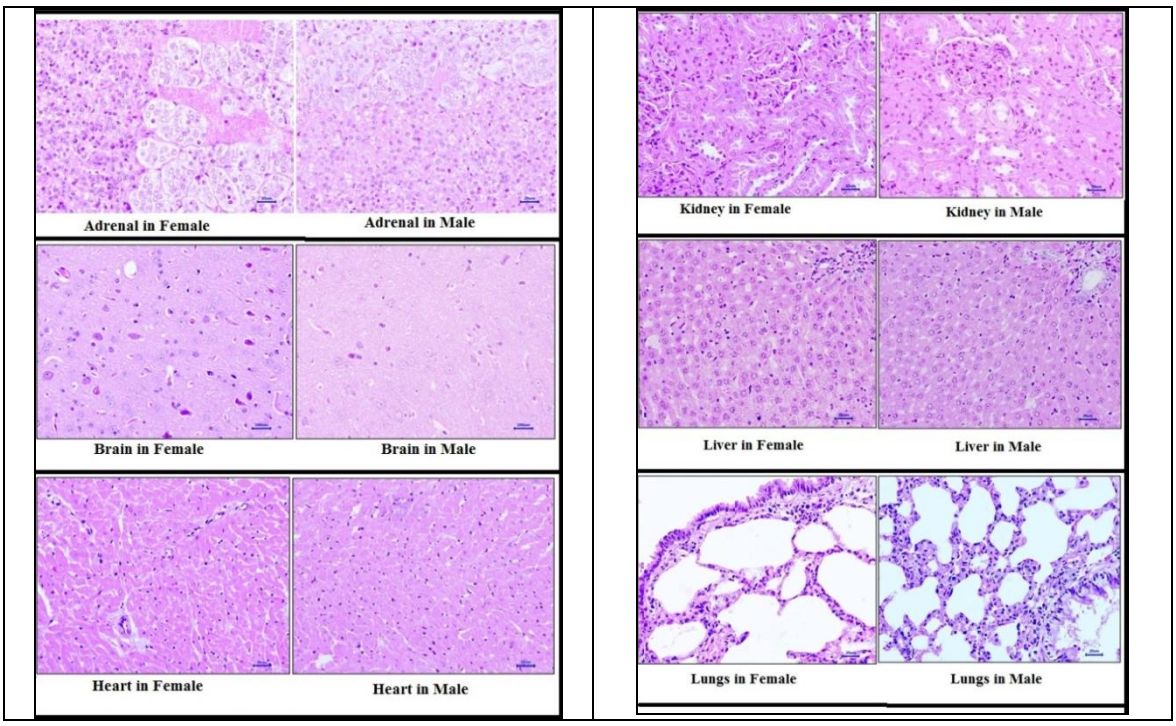
Test Group						
	Total Leukocyte Count (X 10^3/µL)	**RBC Count (X 10^6/µL)**	**Hemoglobin (Hb) (g/dL)**	**Hematocrit (%)**	**MCV (fl)**	**MCH (pg)**
AVG	1.42	6.09	11.34	33.44	55.80	18.88
SD	0.43	0.65	0.66	4.53	11.42	2.78
	Total WBC count (per cu.mm)	**Neutrophils (%)**	**Lymphocytes (%)**	**Eosinophils (%)**	**Monocytes (%)**	**Platelet Count (X 10^3/µL)**
AVG	3840.00	27.00	59.00	2.70	0.94	465.30
SD	598.67	4.17	12.66	1.01	0.27	178.91
Control Group						
	Total	**RBC Count**	**Hemoglobin**	**Hematocrit**	**MCV (fl)**	**MCH**

	Leukocyte Count (X 10^3/µL)	(X 10^6/µL)	(Hb) (g/dL)	(%)		(pg)
AVG	1.67	6.43	11.73	33.14	52.09	19.24
SD	0.52	0.88	0.70	4.16	1.18	3.82
	Total WBC count (per cu.mm)	**Neutrophils (%)**	**Lymphocytes (%)**	**Eosinophils (%)**	**Monocytes (%)**	**Platelet Count (X 10^3/µL)**
AVG	3821.00	31.10	69.00	3.65	1.30	403.70
SD	580.54	4.18	8.56	0.59	0.28	148.40

Table 4: Average of Biochemical Parameters for test and control group

Parameter/ Groups	K (Potassium) mmol/l	Na (Sodium) mmol/l	Glucose Mg/dl	Total Cholesterol Mg/dl	Urea Mg/dl	Creatinine Mg/dl	Total pro g/dl	Alb g/dl	ALT IU/L	AST IU/L
TEST GROUP (Female)										
AVG	4.8	135.8	129.2	55.5	13.3	0.7	71.0	33.7	40.8	153.4
SD	0.6	2.7	19.1	11.8	1.3	0.1	4.4	3.2	6.0	17.9
TEST GROUP (Male)										
AVG	4.66	134.31	130.80	58.61	13.40	0.68	72.13	36.01	40.19	158.94
SD	0.62	3.56	20.14	13.58	0.98	0.11	4.30	3.04	7.76	14.05
CONTROL GROUP (Female)										
AVG	5.0	136.4	129.0	56.0	13.6	0.7	71.7	34.1	41.5	153.6
SD	0.6	2.9	17.9	11.7	1.0	0.1	4.4	3.1	6.7	18.0
CONTROL GROUP (Male)										
AVG	4.78	134.92	131.40	55.31	13.70	0.74	72.47	36.33	40.57	159.49
SD	0.64	4.07	18.30	16.23	0.89	0.11	3.51	3.11	7.40	13.48

Figure 1: Histopathology of Vital Organ of Test Group



CONCLUSION:

Based on the findings of the study and according to the "Globally Harmonized System (GHS) for classification of chemicals which cause acute toxicity, OECD series on testing and assessment, Number 33; Harmonized Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures [ENV/JM/MONO (2001) 6]", the test article NRL20195PNW has to be classified as GHS Category Unclassified for the obligatory labeling requirement for oral toxicity. This category indicates that following acute oral exposure of NRL20195PNW in female mice;the LD_{50} cut-off value is more than 5000 mg/kg body weight.Results of the 28 days repeat dose toxicity indicated that the NRL20195PNW had no adverse effect on general health, growth, behavioral, neurological, Haematological, clinical chemistry and urinalysis parameters, organ weights and gross of the tissues/organs of the mice treated at the dose level of 1000 mg/kg body weight. Based on the findings of this study, the no observed adverse effect level (NOAEL) of NRL20195PNW in mice, following oral administration for 28 days was found to be 1000 mg/kg body weight.

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