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Pre-clinical Safety Evaluation of Recombinant Human Granulocyte Macrophage Colony Stimulating Factor (rh GM-CSF)

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Abstract

Granulocyte-macrophage colony-stimulating factor (GM-CSF), a monomeric glycoprotein produced by macrophages, T cells, mast cells and NK cells naturally. Since, this cytokine is responsible for the survival, proliferation, differentiation and function of myeloid cells, it has been used in vast applications such as cancer and inflammatory diseases. There is huge requirement of this therapeutic drug, recombinant human GM-CSF (rhGM-CSF) has been developed in yeast cells and characterized by various biophysical properties by the present investigators using rDNA technology. The rhGM-CSF has been increased the yield by expressing with T7 promoter and downstream of pelB signal peptide (2.5 kDa) in yeast cells and it can be used as a therapeutic protein where it shows the similar biochemical properties with natural human rhGM-CSF protein (Chitra *et al*., 2017). Further, to characterize any toxicological symptoms that might be produced in animals and to find recommended dose levels of rhGM-CSF for using as a therapeutic drug, the present investigation has been focused. Acute, sub-acute and chronic toxicity tests are routinely carried out by various pharmaceutical companies in the development of new medicines. Initially, acute study was conducted by administering ten times of the therapeutic dose of rhGM-CSF to mice and observed for 15 days to find the pre-terminal morbidity, mortality and any toxic reactions. There are no significant toxicological symptoms or any other adverse reactions observed during the sub chronic study in all males and females, it indicating that rhGM-CSF is safe up to the 325 µg/ 20 g mice and 116.6 µg/kg rabbit dose level. Based on these results, the present investigation suggested rhGM-CSF is safe and further conducting the clinical studies might be helpful for using the rhGM-CSF as a therapeutic drug.

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Keywords

GM-CSF: Granulocyte-macrophage colony-stimulating factor, Acute toxicity, Sub-chronic toxicity study, Recombinant DNA technology, Clinical chemistry, Hematology.

Introduction

Granulocyte macrophage colony stimulating factor (GM-CSF) is a monomeric 23 kD glycoprotein with 4∞-helical bundle structure produced by macrophages, T cells, mast cells and NK cells (Metcalf, 1998). It acts as a cytokine and colony stimulating factor 2 (CSF2) that binds to the heterodimeric receptor, which is belonging to type-1 cytokine receptor family (Zhang, 2000). GM-CSF activates the inflammatory cascade by stimulating

the stem cells to generate granulocytes and monocytes which activates macrophages to an increase rapidly in their numbers to facilitate development of the immune system and promotes defense against infections (Paine *et al*., 2000). This cytokine is responsible for the survival, proliferation, differentiation and function of myeloid cells (Guthridge, 1998). Recently, GM-CSF has been used to regimens for the mobilization of hematopoietic progenitor cells and also essential for maintaining basal hematopoiesis (Fantuzzi, 2003, Cashen *et al*., 2004). Further, the increase in dendritic cells content and activity following local and systemic GM-CSF administration support a role for GM-CSF as an immune stimulant and vaccine adjuvant in cancer patients (Ferlazzo *et al*., 2000; Sanoda, 1988).

Recombinant human GM-CSF (rhGM-CSF) has been developed in yeast cells and characterized by various biophysical properties by the present investigators using recombinant DNA technology (Ballinger *et al*., 2006). The rhGM-CSF has been increased the yield by expressing with T7 promoter and downstream of pelB signal peptide (2.5 kDa) in yeast cells and it can be used as a therapeutic protein where it shows the similar biochemical properties with natural human rhGM-CSF protein (Chitra *et al*., 2017).

Further, to characterize any toxicological symptoms that might be produced in animals and to find recommended dose levels of rhGM-CSF for using as a therapeutic drug, the present investigation has been focused. Acute, sub-acute and chronic toxicity tests are routinely carried out by various pharmaceutical companies in the development of new medicines. Initially, pretesting was conducted by administering rhGM-CSF to mice and observed for 15 days to find the pre-terminal morbidity, mortality and any toxic reactions. There are no toxic signs or pre-terminal morbidity deaths were observed during the study period. Further, 90 days sub-chronic study was conducted in mice to examine the physical, biochemical, hematological and histopathological observations (Schedule Y). There are no significant toxicological symptoms or any other adverse reactions observed during the sub chronic study in all males and females, it indicates that rhGM-CSF is not toxic up to the 325 µg/20 g mice dose level for mice and 116.6 µg/kg rabbit. Despite the various uses over long time periods, no toxicological data is available regarding the safety of repeated exposure to GM-CSF. Further, the conducting the clinical evaluation will be helpful for establishing the rhGM-CSF as a therapeutic drug in market.

Materials and Methods

Preparation of test compound

Test sample

The expressed rhGM-CSF protein was aseptically formulated rhGM-CSF (300mcg/ml) in phosphate buffer, pH 7.0 containing mannitol (20 mg/ml) and sodium chloride (150 mM) followed by lyophilization and stored at 2-8º C. The lyophilized powder was reconstituted in injectable water and diluted in 0.9% normal saline to get the protein solution of required concentration.

Experimental animals

Both sexes of mice (*Mus Musculus*) were used for the acute and sub chronic toxicology studies. Healthy pathogenic free, Swiss albino mice weighing 18-25g, 4-6 weeks old and both sexes of New Zealand White rabbits weighing 1.0 -1.8 Kg (Approval No. BT/BS/1753/2001 PID & BT/BS/1754/2001 PID) were purchased from National Institute of Nutrition (National Center For Laboratory Animal Science, CPCSEA Registered No. 154/1999/CPCSEA Hyderabad, India). All the animals were housed in polypropylene cages in pathogen-free experimental rooms, and were maintained at 22 ± 3 ℃ and relative humidity of 55 ± 6% with 12 h light/dark cycle. During acclimatization, the animals were housed in polycarbonate cages with a standard pellet diet and tap water *ad libitum* and with 10 – 15 air changes per hour in experimental room. All the animals were acclimatized at experimental conditions for 7 days. All experiments were performed in accordance with the Institutional Animal Ethics Committee.

Pre-testing with rhGM-CSF

A short term study was conducted in ten mice (five males and five females) by single dose administration of rhGM-CSF (10 X therapeutic dose, 65 µg/20g mice) intramuscularly (Table 1) at the fore limb and hind limb region of mice on both sides alternatively to evaluate the sensitization of the animal to the test compound. The vehicle control group received 0.9% normal saline without the test compound. Similarly, single administration of 116.6 µg rhGM-CSF per kg rabbit was given intramuscularly. The vehicle control for this study was made by using 0.9% normal saline without the test compound. All the animals were observed for 15 days for pre-terminal morbidity, mortality and any toxic reactions.

Sub chronic toxicity study

Sub-chronic toxicity test was performed by conducting in 80 mice (40 males + 40 females) by administering rhGM-CSF daily for thirty days and observed up to 90 days (Griffths and Lumley, 1997). Both sexes of mice were equally divided into four groups with twenty animals each (10 males and 10 females) viz., Group 1: Vehicle control, Group 2: Therapeutic Dose: 32.5 µg/20 g mice, Group 3: Intermediate Dose: 162.5 µg/20 g mice, Group 4: High Dose: 325 µg/20 g mice. The vehicle control group received for this study was made by using 0.9% normal saline without the test compound. For rabbit, Group 1: Vehicle control, Group 2: Therapeutic Dose: 11.66 µg/kg rabbit, Group 3: Intermediate Dose: 58.3 µg/kg rabbit, Group 4: High dose: 116.6 µg /kg rabbit. The site of injection was cleaned with alcohol swab before and after administering test compound. The test compound as per dosage schedule (Table 1) was administered intramuscularly in the fore limb and hind limb region of mice on both sides alternatively. Sterile disposable syringes of 1ml (with 1/40 graduation) with 30 gauge needles (1/2 inch) were used for injection. The volume of injection did not exceed more than 200 µl. The animals were observed for pre-terminal morbidity, mortality and behavioral throughout experimental period. After the experimental period, the mice were fasted overnight (water allowed) blood samples were collected for hematology and clinical observations after which euthanized by CO_2 chamber and subjected to gross necropsy and the findings recorded. The individual organs were again examined for gross morphology changes after removal. They were examined clinically and blood was collected for clinical chemistry and hematology after anesthetization which was carried out after 48^{th} h and 30^{th} day, 60^{th} day and 90^{th} day after the exposure to the test compound. After gross necropsy, organ weights (GM 6101, Sartorious) as well as histopathological examination of various organs such as liver, spleen, kidneys, brain, thymus and injection site, was carried out on day 90^{th} after the last exposure to the test compound. The tissues were sliced adequately wherever necessary. After a minimum of 24 h fixation, they were sampled and processed by conventional methods, paraffin blocks were made and 6 µm paraffin sections were stained with Hematoxylin and Eosin. They were examined under a light microscope. All deviations from normal histology were recorded and compared with corresponding controls. Also, the potential production of neutralizing antibodies was studied.

All the test systems were observed for mortality, live phase of animals (Food intake, body weight, general behavior, Water intake), Cage side observation (Home cage activity, Feces excretion, Urine excretion), Physical examination (Hair Coat, Piloerection, lacrimation, Salivation, Respiration rate and Character, Eye prominence and Eyelid colosure, Convulsion, biting, tremors), Neurological examination (Locomotor activity, rearing activity, tail elevation, abnormal gait, Head position, Pinna touch response), qualitative Urine examination (Urobilinogen, Protein, P^H, RBC, Gravity, ketone, Bilirubin, Gluose) were qualitatively tested using Ames Multistix reagent strips at the time intervals, before exposure to test compound and Post Exposure after the exposure to the test compound (Urine collection was done for 12 hours before testing). Clinical chemistry (glucose, creatinine, total protein, ALP, SGOT, SGPT, bilirubin, potassium, magnesium and calcium) were estimated using Alfa Biotech PLD 951 auto-analyzer, supplied by Wipro Biomed. All analytical kits were purchased from Recombigen Laboratories Pvt Ltd, New Delhi. Hematology parameters (WBC, RBC, Hgb, HCT, MCV, MCH, MCHC, Platelet Count, and MPV) were estimated by using Beckman Coulter Counter.

Statistical analysis

Results are expressed as Mean ± standard error mean (SEM). Data obtained was analyzed by using one way ANOVA followed by Dunnett's test and $p<0.05$ was considered as statistically significant. Treatment groups were compared with vehicle control by fisher's exact or Chi-square test. Between group comparisons by means of Kruskal-Wallis one-way ANOVA and individual group comparisons by Mann-Whitney U test (test groups with vehicle control) were the method of analysis. Heterogeneity of variance was tested by Levene's statistic. After confirming homogeneity, ANOVA linear models were done for between group significant F-ratio. Post-hoc tests by means of Tukey's HSD were carried out for comparison of test groups with vehicle control. Statistical analysis was carried out on SAS 8.2 version.

Results and Discussion

Pre-testing with rhGMCSF (Acute toxicity study)

Pre-testing was conducted with the rhGMCSF (10X) alone administered topically using ten mice (5M + 5F) showed no toxic signs or pre-terminal morbidity deaths during the study period. The LD_{50} for the mice was found to be

greater than 325 µg and greater than 116.6 µg for rabbit respectively.

Sub chronic toxicity study

There were no treatment related toxicity signs and mortality observed in both sexes of mice treated at 32.5 µg/20 g mice, 162.5 µg/20 g mice and 325 µg/20 g mice intramuscularly during the 90 days observation period. Similarly, there were no treatment related toxicity signs and mortality observed in both sexes of rabbit 11.66 µg/kg rabbit 58.3 µg/kg rabbit 116.6 µg/kg rabbit treated intramuscularly during the 90 days observation period. There is no mortality and morbidity in all the groups during the experimental period. The water and food intake was also not affected by the administration of rhGMCSF and these were similar when compared to vehicle control group animals. The food intake by mice at the initial stages was between 3-5 g and it increased to 4-6 g on an average in all the groups of animals at the end of the study period (Data was not shown) There were no abnormalities in the head and limb positions, gait, locomotor activity, rearing activity, tail elevations, pinna touch response, fecal consistency and urine color and amount, behavior, activeness, piloerection, lacrimation, salivation, respiration rate, eye prominance, eye lid closure in all treated group animals as wells as vehicle control group animals during the experimental period. There is no convulsions, aggressiveness, biting character and tremors observed in all the groups.

Body weight

The gain in body weight was normal (10-12 g mice and 1.5 kg rabbit) in all groups of animals during the study period. There is no significant ($p \leq 0.05$) treatment effect on the body weights of male and female mice exposed to test compound at various levels was seen as compared to vehicle control group.

Clinical chemistry

There were no abnormalities in clinical chemistry parameters *viz* blood glucose, total protein, SGOT, SGPT, ALP, total bilirubin, creatinine and electrolytes (Magnesium, Potassium and Calcium) in mice treated with rhGMCSF at 48th hour, 30, 60 and 90th days when compared with vehicle control group animals. All the parameters observed in vehicle control group animals as well as treated animals were within the physiological range (Table 2). Further, there were no significant differences in the hematological parameters in all treated animals (Table 5).

Hematology

There were no anomalies observed in hematology parameters including WBC, RBC, platelets, haemoglobin, HCT, MCV, MCH and MCHC at 48th hour, 30, 60 and 90th days in all rhGMCSF treated groups ($p \leq 0.05$) comparing with respective vehicle controls (Table 3 & 4).

Urine analysis

The pH, specific gravity, glucose, protein, bilirubin, urobilnogen and ketone levels were not affected at 48th hour, 30th day, 60th day and 90th day qualitatively in mice treated with rhGMCSF when compared with vehicle control group animals.

Organ weights

There were no gross changes in all the organs in all treated group animals and also no significant differences between various organ weights including kidney, liver, spleen and brain collected from all the treated animals with rhGMCSF ($p \leq 0.05$) (Table 5).

Histopathology

There were no histopathological changes in all the major organs examined, liver, spleen, kidneys, brain, thymus observed in all the test groups as compared to vehicle control group (Data was not shown).

The rhGMCSF has been used for the treatment of malignancies in patients since the rhGMCSF is able to shorten the time period of neutropenia after chemotherapy and to reduce neutropenia-related morbidity such as infections (Schulz *et al*., 1991). Further it has many applications while using as therapeutic drug for many diseases such as acute myeloid leukemia (Lanza *et al*., 1997), solid tumors (de Carren *et al*., 1997), lung cancer (Gautier *et al*., 1995).

Since, rhGMCSF has vast applications in treatment of various diseases and cost effective, the recombinant GMCSF has been developed in *E.coli* which has shown similar properties with the natural sources (Chitra *et al*., 2017) and further the pre-clinical toxicology study was conducted before trail the clinical applications.

Table.1 rhGMCSF administration to mice in acute and sub-chronic toxicity study

S. No	Study Details	Animal Group	rhGMCSF administration	Dosage Duration
1	Acute	Vehicle Control	Formulation buffer	Once
		Test group (10X)	325µg/ 20 g mice	
			116.6 µg/kg rabbit	
2	Sub Chronic Study	Vehicle Control	Formulation buffer	30 doses, once daily
		Therapeutic Dose (1X)	32.5 µg/ 20 g mice	
			11.66 µg/kg rabbit	
		Intermediate Dose (5X)	162.5 µg/ 20 g mice	
			58.3 µg/kg rabbit	
		High Dose (10X)	325 µg/ 20 g mice	
			116.6 µg/kg rabbit	

Table.6 Rabbit organ weights at 90th day

	Rabbit					**Mice**			
Parameters	**Groups (Average of 10 Rabbits)**					**Groups (Average of 20 Mice)**			
	VC	**TD**	**ID**	**HD**		**VC**	**TD**	**ID**	**HD**
Spleen	0.86±0.09	0.88±0.10	0.96 ±0.08	0.96±0.08		0.110±0.044	0.100±0.00	0.100±0.00	0.100±0.00
Heart	4.06±0.09	4.05±0.15	4.00±0.00	4.05±0.15		0.100±0.00	0.100±0.00	0.100±0.00	0.100±0.00
Liver	51.17±4.73	53.50±1.89	54.75±0.45	53.37±2.08		1.36±0.07	1.27±0.11	1.20±0.075	1.24±0.068
Kidney	11.08±0.87	12.55±0.59	13.20±0.63	13.32±0.96		0.42±0.052	0.415±0.048	0.420±0.52	0.375±0.055
Brain	6.49±0.17	6.35±0.33	6.090±0.16	6.345±0.32		0.375±0.044	0.350±0.051	0.390±0044	0.375±0.044

Table.2 Sub acute toxicity study - Clinical chemistry parameters in mice administered with rhGMCSF

Parameter	Vehicle control				Therapeutic Dose				Intermediate Dose				High Dose			
Duration	**48 h**	**30 d**	**60 d**	**90 d**	**48 h**	**30 d**	**60 d**	**90 d**	**48 h**	**30 d**	**60 d**	**90 d**	**48 h**	**30 d**	**60 d**	**90 d**
Blood Glucose (mg/dl)	67.20 ± 3.28	71.52 ± 7.97	78.54 ± 14.0	71.27 ± 6.50	70.98 ± 2.44	77.75 ± 10.03	76.76 ± 12.5	74.86 ± 6.13	69.64 ± 4.40	81.83 ± 9.92	79.65 ± 9.15	77.39 ± 6.34	71.45 ± 3.31	82.18 ± 9.05	80.52 ± 1.97	77.72 ± 6.93
Total Protein (gm/dl)	2.16 ± 0.15	2.24 ± 0.08	2.34 ± 0.25	2.25 ± 0.11	2.27 ± 0.14	2.18 ± 0.16	2.43 ± 0.23	2.28 ± 0.19	2.27 ± 0.26	2.30 ± 0.17	2.21 ± 0.15	2.25 ± 0.20	2.29 ± 0.20	2.37 ± 0.15	2.26 ± 0.23	2.25 ± 0.20
SGOT (IU/L)	33.34 ± 5.43	33.60 ± 3.39	32.66 ± 4.70	36.14 ± 2.55	32.51 ± 5.0	34.71 ± 5.08	34.45 ± 3.31	36.72 ± 4.31	35.68 ± 5.58	34.95 ± 2.64	34.30 ± 2.88	36.24 ± 3.22	36.16 ± 2.65	34.93 ± 3.48	35.97 ± 2.27	35.30 ± 3.38
SGPT (IU/L)	21.84 ± 3.61	21.38 ± 4.05	21.53 ± 3.53	23.79 ± 3.77	21.63 ± 3.84	23.46 ± 6.82	23.01 ± 3.72	25.50 ± 3.76	24.31 ± 4.02	24.77 ± 6.73	23.21 ± 4.54	24.22 ± 5.21	24.18 ± 4.54	24.53 ± 5.57	23.58 ± 3.23	23.96 ± 3.17
ALP (IU/L)	40.14 ± 5.83	42.17 ± 6.42	39.38 ± 4.39	40.54 ± 3.66	37.60 ± 5.10	42.85 ± 6.00	40.22 ± 4.48	40.58 ± 4.12	36.46 ± 2.94	39.09 ± 5.50	39.06 ± 4.51	39.96 ± 3.17	34.37 ± 3.58	36.18 ± 3.25	38.31 ± 2.81	40.02 ± 2.85
Total bilirubin (mg/dl)	0.39 ± 0.28	0.35 ± 0.28	0.350 ± 0.31	0.329 ± 0.19	0.35 ± 0.17	0.53 ± 0.30	0.37 ± 0.22	0.36 ± 0.14	0.29 ± 0.09	0.43 ± 0.19	0.361 ± 0.25	0.39 ± 0.18	0.31 ± 0.14	0.370 ± 0.14	0.388 ± 0.17	0.41 ± 0.16
Triglycerides	92.19 ± 10.7	91.58 ± 8.70	90.85 ± 8.62	92.74 ± 10.10	91.57 ± 9.38	92.34 ± 12.44	90.51 ± 11.07	93.17 ± 10.16	90.28 ± 8.78	91.73 ± 10.72	91.29 ± 10.18	94.28 ± 11.09	90.87 ± 11.54	90.20 ± 12.0	92.90 ± 8.99	94.12 ± 9.00
Cholesterol	77.93 ± 9.01	79.28 ± 9.28	75.86 ± 7.50	75.53 ± 7.22	79.50 ± 8.20	76.33 ± 5.23	72.22 ± 5.24	74.49 ± 4.41	77.60 ± 6.78	74.74 ± 6.53	72.47 ± 3.08	74.97 ± 7.21	73.51 ± 8.27	72.21 ± 7.64	70.26 ± 6.97	70.56 ± 6.14
Magnesium (m.mol/L)	1.742 ± 0.247	1.56 ± 0.311	1.69 ± 0.26	1.570 ± 0.219	1.74 ± 0.102	1.50 ± 0.370	1.81 ± 0.102	1.729 ± 0.237	1.76 ± 0.064	1.465 ± 0.445	1.800 ± 0.056	1.748 ± 0.150	1.804 ± 0.04	1.55 ± 0.332	1.688 ± 0.29	1.818 ± 0.076
Potassium (m.mol/L)	5.02 ± 0.603	4.96 ± 0.640	4.98 ± 0.873	5.10 ± 0.908	5.134 ± 0.53	5.105 ± 0.692	5.29 ± 0.731	5.40 ± 0.61	5.00 ± 0.772	4.96 ± 0.691	5.04 ± 0.928	5.177 ± 0.416	5.05 ± 0.577	5.114 ± 0.788	5.22 ± 1.04	5.05 ± 0.957
Urea (mg/dl)	14.23 ± 2.09	16.26 ± 1.15	15.45 ± 2.83	15.31 ± 2.61	13.25 ± 1.73	14.85 ± 2.32	16.70 ± 3.05	16.90 ± 2.93	12.57 ± 3.04	14.88 ± 1.95	17.80 ± 3.85	17.59 ± 4.55	11.44 ± 1.50	16.74 ± 2.50	16.95 ± 2.37	17.00 ± 1.31

Table.3 Hematological report of Mice for WBC, RBC, Hgb and HCT (%)

Days	Vehicle control				Therapeutic Dose				Intermediate Dose				High Dose			
Parameters	WBC (x 03µl)	RBC (x 0^6µl)	Hgb (g/dl)	HCT (%)	WBC (x 03µl)	RBC (x 0^6µl)	Hgb (g/dl)	HCT (%)	WBC (x 03µl)	RBC (x 0^6µl)	Hgb (g/dl)	HCT (%)	WBC (x 03µl)	RBC (x 0^6µl)	Hgb (g/dl)	HCT (%)
48 Hrs	11.70± 1.36	10.06± 0.42	14.92± 1.72	46.7±2 .50	10.91± 1.72	10.11± 0.51	14.95± 1.70	47.21± 2.65	9.87± 1.36	10.12± 0.23	15.97± 0.38	47.88± 1.35	10.17± 1.92	10.69± 1.85	15.3±1 .62	44.47± 10.17
30 days	11.66± 2.49	9.46±0 .40	14.67± 0.70	44.41± 1.73	9.88±4 .02	9.35±0 .29	14.70± 1.11	44.46± 2.59	8.81± 2.42	9.75± 1.00	15.86± 0.81	48.35± 2.62	7.64±3 .06	9.963± 0.67	15.8±0 .57	47.17± 1.80
60 day	8.79±2 .19	10.04± 0.56	15.85± 0.84	47.4± 2.62	8.01±2 .84	10.12± 0.24	15.09± 2.35	48.12± 2.42	6.36± 1.38	10.61± 0.41	16.78± 0.72	50.06± 2.00	7.64±2 .36	10.39± 0.54	16.5±0 .67	48.52± 1.81
90day	6.96±1 .92	10.21± 0.45	16.34± 0.76	48.39± 1.59	7.68±2 .84	10.39± 0.47	16.35± 0.69	47.99± 1.95	6.82± 2.98	10.65± 0.80	17.47± 1.00	50.86± 3.22	6.67±3 .41	10.71± 0.41	16.97± 0.67	50.44± 2.02

Days	Vehicle control				Therapeutic Dose				Intermediate Dose				Days			
Parameters	WBC(x 03µl)	RBC (x 0^6µl)	Hgb (g/dl)	HCT (%)	WBC (x 03µl)	RBC (x 0^6µl)	Hgb (g/dl)	HCT (%)	WBC (x 03µl)	RBC (x 0^6 µl)	Hgb (g/dl)	HCT (%)	WBC (x 03µl)	RBC (x 0^6µl)	Hgb (g/dl)	HCT (%)
48 Hrs	4.24± 0.62	15.97± 3.16	12.82± 0.67	41.16± 0.90	4.36± 0.20	15.67± 1.71	12.56± 1.20	39.2±1 .86	6.32± 1.30	14.76± 1.81	12.56± 0.55	13.30± 0.86	4.28± 0.32	13.97± 1.71	12.40± 0.55	39.34± 1.61
30 days	4.34± 0.39	13.21± 1.80	12.32± 0.57	39.20± 1.81	4.62± 0.29	15.07± 2.08	12.34± 2.59	41.1±5 .06	4.52± 0.35	14.19± 1.69	11.92± 0.91	39.50± 2.39	4.18± 0.26	13.74± 1.89	12.68± 1.16	40.82± 3.90
60 day	3.70± 0.33	13.72± 1.53	12.94± 1.57	40.48± 3.50	4.22± 0.23	14.62± 1.21	13.16± 0.82	41.6±2 .35	3.98± 0.36	13.85± 1.30	11.94± 0.82	38.72± 2.29	3.92± 0.54	15.09± 1.64	11.94± 0.82	39.48± 3.37
90day	4.45± 0.36	13.81± 1.41	12.56± 1.11	40.88± 3.45	4.36± 0.18	13.10± 1.36	13.44± 1.32	41.58± 3.45	4.47± 0.20	13.28± 0.95	12.56± 0.55	40.60± 2.33	4.52± 0.40	13.92± 1.83	13.70± 0.75	42.30± 3.27

Table.4 Hematological report of Rabbits for MCV, MCH, MCHC and Plt

Days	Vehicle control				Therapeutic Dose				Intermediate Dose				High Dose			
Parameters	MCV fL	MCH pg	MCHC	Plt (x 10^3µl)	MCV fL	MCH pg	MCHC	Plt (x 10^3µl)	MCV fL	MCH pg	MCHC	Plt (x 10^3µl)	MCV fL	MCH pg	MCHC	Plt (x 10^3µl)
48 hours	64.79 ± 3.03	21.34 ± 1.05	31.63 ± 1.34	283.8 ± 51.62	65.56 ± 3.77	21.56 ± 1.40	31.35 ± 0.88	209.800 ± 141.15	64.06 ± 1.82	20.46 ± 0.86	30.87 ± 0.84	176.40 ± 81.79	62.20 ± 2.42	20.48 ± 1.51	30.90 ± 1.29	216.803 ± 92.23
30 days	66.06 ± 2.09	20.74 ± 0.52	31.42 ± 0.69	238.40 ± 112.7	67.40 ± 2.61	21.28 ± 0.68	31.58 ± 0.24	266.600 ± 179.44	65.80 ± 1.97	20.26 ± 0.75	30.80 ± 0.62	205.80 ± 52.60	65.80 ± 2.70	20.48 ± 1.06	31.14 ± 1.01	265.80 ± 91.21
60 day	66.62 ± 2.61	21.26 ± 0.95	31.92 ± 1.26	206.40 ± 85.96	68.04 ± 4.13	21.48 ± 1.33	31.58 ± 0.35	219.0 ± 71.68	65.26 ± 1.70	20.34 ± 0.73	31.18 ± 1.05	210.20 ± 41.79	63.94 ± 2.32	20.14 ± 0.90	31.48 ± 0.34	221.000 ± 112.47
90 day	65.96 ± 2.87	20.24 ± 0.78	30.71 ± 0.91	181.20 ± 21.11	68.14 ± 1.92	22.02 ± 1.77	32.28 ± 1.67	216.000 ± 80.33	67.08 ± 1.21	21.34 ± 0.82	31.72 ± 0.95	214.600 ± 36.70	67.12 ± 2.17	21.78 ± 0.42	32.46 ± 1.39	252.600 ± 23.38

Table.5 Hematological report of mice for MCV, MCH, MCHC and Plt

Days	Vehicle control				Therapeutic Dose				Intermediate Dose				High Dose			
Parameters	MCV fL	MCH pg	MCH C	Plt (x 103µl)	MCV fL	MCH pg	MCH C	Plt (x 103µl)	MCV fL	MCH pg	MCH C	Plt (x 103µl)	MCV fL	MCH pg	MCH C	Plt (x 103µl)
48 hours	46.41 ±1.26	15.41 ±0.38	33.21 ±0.67	800.50± 245.72	46.66 ±1.55	15.51 ±0.42	33.13 ±0.69	984.690 ±241.74	47.29 ±1.48	15.80 ±0.50	33.3± 0.38	826.49± 250.3	47.31 ±1.68	18.51 ±8.86	31.21 ±0.59	847.973 ±168.16
30 days	46.97 ±1.16	15.52 ±0.45	33.04 ±0.50	757.50± 176.64	47.44 ±2.32	15.65 ±0.99	33.03 ±0.61	1066.80 ±356.46	48.86 ±4.21	16.07 ±1.20	33.13 ±0.96	1085.80 ±151.69	47.17 ±1.37	15.79 ±0.62	33.53 ±0.60	895.50± 211.31
60 day	47.03 ±0.82	15.68 ±0.40	33.32 ±1.23	1155.60 ±210.81	47.54 ±1.61	16.12 ±0.55	33.95 ±0.52	1195.60 ±252.85	47.29 ±1.07	15.78 ±0.50	33.52 ±0.31	1157.40 ±224.48	46.80 ±1.82	15.95 ±0.75	34.05 ±0.71	1337.0± 133.88
90	47.37 ±1.44	16.00 ±0.60	33.76 ±0.75	1073.94 ±438.94	46.22 ±1.96	15.75 ±0.70	34.08 ±0.50	1280.50 ±248.08	47.79 ±1.32	16.40 ±0.60	34.35 ±0.58	1128.50 ±219.81	47.08 ±1.89	15.83 ±0.47	33.70 ±0.65	1154.10 ±271.02

In present investigation, the Acute and sub-chronic effects of rhGMCSF in mice as well as rabbits were presented. It has been observed that there are no toxicological symptoms in both acute and sub-chronic toxicity at pre-defined dosage levels in all the treated groups in both mice and rabbits. Based on the acute toxicity study for 14 days, it was found that LD_{50} in mice was 325 µg/20 g mice.

Further, the sub chronic toxicity test in mice was conducted after the observation of non-lethality of single dose of 325 µg rhGM-CSF/20 g mice in acute toxicity study. 30 day repeated dose toxicology study with recovery groups revealed the safety profile of rhGM-CSF. Taking this into consideration we studied the safety profile of the rhGM-CSF for longer duration of exposure, *i.e.*, 90 day subchronic toxicity study. The animals exposed to the rhGMCSF during 90 day sub-chronic toxicity study did not show any abnormalities in behavior and physical activities. The gain in body weights, between exposed animals to rhGMCSF and respective control group animals was not significant (Figure 1).

In addition, qualitative tests in urine also did not shown any significant abnormalities. Further, blood glucose, protein levels, kidney and liver function tests including electrolytes (Calcium, Magnesium and Potassium) were found to be in normal range and no significant changes observed in various group of animals exposed to the rhGMCSF and vehicle control. The hematological parameters were also found to be within normal range immediately after exposure and post exposure to test compound (Tables 3 and 4) in both mice and rabbits. There were no histopathological changes in various organs viz. heart, lung, brain and kidney in the mice exposed to the rhGMCSF. Based on the all these results, it has been revealed that there are no abnormalities in behavior, clinical, hematological or immunopathological and histopathological observations at all recommended therapeutic dose and higher doses under the experimental conditions. Based on these results, the present investigation suggested rhGM-CSF is safe and further conducting the clinical studies might be helpful for using the rhGM-CSF as a therapeutic drug.

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