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Acute and Long Term Safety Evaluation of Zincovit Syrup (Nutritional Food Supplement) In Wistar Rats.

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ABSTRACT

The aim of the present study was to investigate the acute and sub-chronic toxicity associated with Zincovit syrup (Nutritional food supplement) in Wistar rats. Acute toxicity class method (OECD 423 guideline) was employed to determine acute toxicity in Wistar rats. Animals were observed individually after dosing daily for a total of 14 days. Sub chronic toxicity was investigated in normal control (2% gum acacia, 1 ml/kg/day) and Zincovit syrup at 15, 60 and 240 mg/kg/day individually for 3 months in adult female Wistar rats (4 groups, n= 6). Clinical signs, hematological and biochemical parameters were assessed. During the acute toxicity study, according to annex 2a of OECD 423 guidelines, Zincovit syrup falls under Category 4 (>300-2000) of globally harmonized classification system (GHS). For Zincovit syrup, LD_{50} cut-off among Wistar rats was observed at 1000 mg/kg. There was no significant change in their body weight. During the 90 days of sub-chronic toxicity study, treatment with Zincovit syrup among Wistar rats, the lowest-observed-adverse-effect level (LOAEL) and no-observed-adverse-effect level (NOAEL) was observed at 60 mg/kg/day and 15 mg/kg/day respectively. The present study demonstrated the long term safety of Zincovit syrup at its low dose (15 mg/kg/day) in Wistar rats.

Keywords: Zincovit syrup, Lysine, Multivitamin-multimineral nutritional food supplement, Acute toxicity, Sub chronic toxicity, Antioxidants.

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INTRODUCTION

Assessment of the safety of nutrients presents a challenge different from that posed by the assessment of other chemicals in food such as additives or contaminants. Because nutrients are essential, a dose response relation exists at both ends of the intake range, separated by a safe range of intake that reflects normal homeostatic processes. The safe intake may not be the same for all population groups and life stages. The size of the safe intake range for each nutrient will vary and in a few cases may be very small. Usually, multivitamin and mineral supplements are used to ensure adequate intake and to prevent or mitigate diseases. An update of the data on adverse effects will help researchers to evaluate the appropriateness of upper intake level of these nutrients. Zincovit syrup is a combined formulation of vitamins, minerals and lysine. Lysine, or L-lysine, is an essential amino acid. Lysine is important for proper growth, and it plays an essential role in the production of carnitine, a nutrient responsible for converting fatty acids into energy and helping to lower cholesterol. Lysine appears to help the body to absorb calcium, and it plays an important role in the formation of collagen, a substance important for bones and connective tissues including skin, tendon, and cartilage. Zincovit syrup releases a stream of anti-oxidant benefits [1]. In another study, we have reported the acute and long term safety of combined formulation of grape seed extract and Zincovit tablets where flaxseed oil and lysine were not the ingredients [2]. Therefore, the present study was aimed to evaluate the acute and long term safety of orally administered Zincovit syrup in Wistar rats.

MATERIALS AND METHODS

Drugs and Reagents

Zincovit syrup was obtained from Apex Laboratories Private Ltd., Chennai (India). The diagnostic kits for alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and creatinine were procured from Aspen Laboratories, New Delhi (India). Sodium chloride and all other chemicals were purchased from Merck Chemicals, Mumbai (India). The reagents were equilibrated at room temperature for 30 minutes before use, either at the start of analysis or when reagent containers were refilled.

Animals

Adult female Wistar rats (4 to 6-weeks-old), nulliparous and non-pregnant were selected for the study, which were bred locally in the central animal house of Manipal University, Manipal. Animals were housed in separate polypropylene cages (3 animals in each cage). They were maintained under standard conditions with temperature (22–24°C), 12-h light/12-h dark cycle and relative air humidity 40–60%. The animals were acclimatized to the laboratory conditions for one week before the start of the experiment. The animals were provided with a normal pellet diet (VRK Nutritional Solutions, Pune, India) and water ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC/KMC/42/2013) and experiments were conducted according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

Experimental design

Acute toxicity study

For acute toxicity study, both rats were divided individually into four groups (n= 3).

Group IA : 5 mg/kg of Zincovit syrup
Group IB : 5 mg/kg of Zincovit syrup
Group IIA : 50 mg/kg of Zincovit syrup
Group IIB : 50 mg/kg of Zincovit syrup
Group IIIA : 300 mg/kg of Zincovit syrup
Group IIIB : 300 mg/kg of Zincovit syrup
Group IVA : 2000 mg/kg of Zincovit syrup
Group IVB : 2000 mg/kg of Zincovit syrup

The acute toxicity class method (OECD 423) was employed for the acute toxicity study of Zincovit syrup [3]. In this method, Zincovit syrup was administered through oral route as a single dose to a group of experimental animals and a sequential testing procedure with three animals per group was used. Food was withheld over-night prior to dosing of test drug (water ad libitum). The body weight of each animal was noted and the respective dose of drug was administered. After the administration of Zincovit syrup, food was withheld for 3-4 hours. Treatment of animals at the next dose was delayed until the confirmation of survival of the previously dosed animals. Animals were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with more attention given during the first 4 hours and daily thereafter, for a total of 14 days. Observations included were changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, somatomotor activities and behavior patterns. Tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma were the prime focus. At the end of the test surviving animals were weighed and rehabilitated.

Sub chronic toxicity study

For sub chronic toxicity study, rats were divided into 4 groups (n=6). The corresponding doses of drugs were administered orally till 90 days as follow-

Group I : Normal control (2% gum acacia, 1 ml/kg)
Group II : 15 mg/kg/day of Zincovit syrup
Group III : 60 mg/kg/day of Zincovit syrup
Group IV : 240 mg/kg/day of Zincovit syrup

Collection of blood samples

At the end of the experimental period, the animals were anesthetized with ketamine (80 mg/kg; *i.p.*) following a 12 h fast. Blood was collected from retro-orbital plexus through capillary tube. Serum was obtained by centrifugation of blood at 3,000 rpm for 20 min at 4°C using a refrigerated centrifuge (MIKRO 22R, Andreas Hettich GmbH & Co. KG, Germany).

Hematological parameters

0.5 ml of blood from each animal was collected in an EDTA containing vacutainer. Further RBC, WBC, differential leukocytes, platelet count and amount of hemoglobin was measured by veterinary automatic blood cell counter.

Biochemical parameters

Blood glucose level was estimated in the fasting blood samples by glucose oxidase-peroxidase reactive strips (Accu-chek, Roche Diagnostics, USA). Serum was analyzed further for assay of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), creatinine, triglyceride (TG), total cholesterol (T-CHO) and high density lipoprotein cholesterol (HDL-C) according to the standard protocols given along with respective kits (Aspen Laboratories, New Delhi, India). Low-density lipoprotein cholesterol (LDL-C) and Very low-density lipoprotein cholesterol (VLDL-C) was calculated by using Friedewald's equation:

$$VLDL\text{-}C = Triglycerides\ (TG)/5$$

$$LDL\text{-}C = Total\ cholesterol - (HDL\text{-}C + VLDL\text{-}C)$$

Statistical analysis

Using Statistical Package for the Social Sciences (SPSS version 20.0; SPSS Inc., Chicago, USA), data was represented as mean ± standard deviation and analyzed by one way analysis of variance (ANOVA) followed by post hoc Tukey test. A level for $p \leq 0{\cdot}05$ was considered to be statistically significant.

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RESULTS

During acute toxicity study: A total of 2 rats died at the dose of 2000 mg/kg (Group IV A). According to annex 2a of OECD 423 guidelines, Zincovit syrup falls under Category 4 (>300-2000) of Globally Harmonized Classification System (GHS). For Zincovit syrup, LD50 cut-off among Wistar rats was observed at 1000 mg/kg.

During sub chronic study: There was no significant change in count of WBC, RBC, monocyte %, hemoglobin amount, fasting blood glucose level, serum HDL, AST, ALP and body weight of experimental animal groups treated with Zincovit syrup at three different doses (15, 60 and 240 mg/kg) when compared to normal control group of rats. Mostly, in rats treated with Zincovit syrup at the dose of 240 mg/kg, different significant levels were observed for lymphocyte %, granulocyte %, platelet count, serum total triglyceride, total cholesterol, LDL, VLDL, ALT and creatinine in comparison with normal control rats (Table 1-3). During whole body gross necroscopy, there were no significant structural changes observed among normal control rats and rats treated with three different doses of Zincovit syrup (15, 60 and 240 mg/kg). Also, at the end of the treatment, all the experimental animals were found healthy as shown by the normal appearance of respiratory pattern, color of body surfaces, frequency and nature of movement and absence of symptoms like seizures, loss of reflex.

Table 1: Effect of Zincovit syrup on lymphocyte percentage, granulocyte percentage and platelet count

Group (n=6)	Lymphocyte %	Granulocyte %	Platelet count ($x10^3$cells/µl)
I- Normal control	78.65±2.04	10.90±1.36	600.66±27.93
II- Zincovit syrup 15 mg/kg	72.31±3.61	16.15±2.57	340.66±62.62**
III- Zincovit syrup 60 mg/kg	76.43±0.95	13.80±0.85	564.66±30.05
IV- Zincovit syrup 240 mg/kg	69.63±1.17*	18.11±0.66*	408.00±59.27

n, number of rats in each group; ***indicates statistically significant difference compared with normal control (p< 0.001), **indicates statistically significant difference compared with normal control (p<0.01), *indicates statistically significant difference compared with normal control (p< 0.05).

Table 2: Effect of Zincovit syrup on serum total triglyceride, serum total cholesterol, serum VLDL and serum LDL

Groups (n=6)	Serum total triglyceride (mg/dl)	Serum total cholesterol (mg/dl)	Serum VLDL (mg/dl)	Serum LDL (mg/dl)
I- Normal control	106.68±13.64	81.89±4.32	21.33±2.73	22.76±5.13
II- Zincovit syrup 15 mg/kg	104.57±1.66	79.23±3.93	20.91±0.33	18.57±4.96
III- Zincovit syrup 60 mg/kg	153.17±2.74**	90.42±3.62	30.63±0.54**	18.18±1.43
IV- Zincovit syrup 240 mg/kg	155.88±6.03**	108.55±2.97***	31.17±1.20***	42.23±3.23*

n, number of rats in each group; ***indicates statistically significant difference compared with normal control (p< 0.001), **indicates statistically significant difference compared with normal control (p<0.01), *indicates statistically significant difference compared with normal control (p< 0.05).

Table 3: Effect of Zincovit syrup on serum creatinine and serum ALT level

Group (n=6)	Serum Creatinine (mg/dl)	Serum ALT level (U/L)
I- Normal control	0.95±0.11	51.83±6.05
II- Zincovit syrup 15 mg/kg	0.90±0.04	69.88±2.96*
III- Zincovit syrup 60 mg/kg	0.91±0.04	71.77±3.71*
IV- Zincovit syrup 240 mg/kg	0.56±0.05**	77.40±2.65***

n, number of rats in each group; ***indicates statistically significant difference compared with normal control (p< 0.001), **indicates statistically significant difference compared with normal control (p<0.01), *indicates statistically significant difference compared with normal control (p< 0.05).

DISCUSSION

The results of acute toxicity study indicate the LD_{50} of the Zincovit syrup at 1000 mg/kg/day. In the acute toxicity study mortality was observed in Group IVA where overall 2 rats died at the dose of 2000 mg/kg/day. Various biochemical parameters were thoroughly studied in the sub-chronic toxicity study of a period of 90 days. No mortality was observed during this period. Also in the study of hematological parameters

there was no significant change in RBC count, WBC count, monocyte %, hemoglobin amount and fasting blood glucose level while in the rats treated with Zincovit syrup at the dose of 240 mg/kg/day, different significant levels were observed for lymphocyte count, granulocyte count and platelet count when compared to normal control group of rats. Serum biochemical parameters related to hepatic function namely serum HDL, AST, ALP did not show significant changes with Zincovit syrup treated rats compared to normal control group while at the dose 240 mg/kg/day of Zincovit syrup significant changes were observed for levels of serum total triglyceride, total cholesterol, LDL, VLDL, ALT and creatinine in comparison with normal control rats. Serum AST and ALT are the most sensitive hepatic markers employed in the diagnosis of hepatic damage [4]. ALT is primarily localized to the liver and AST is present in a wide variety of tissues like the heart, skeletal muscle, kidney, brain and liver [5]. However, ALT is more specific to the liver and is thus a better parameter for detecting liver injury [6]. In the present study there was significant increase in the level of ALT in all the three dose treatment groups of Zincovit syrup (15, 60 and 240 mg/kg/day) which indicate hepatotoxic potentiality after 90 days dosing. There was no adverse effect on serum creatinine level which shows no interference with filtration process of kidney. At the dose of 240 mg/kg/day significant decrease in serum creatinine level which has no profound clinical significance is probably due to severe hepatic damage [7]. There was a significant increase in serum total triglyceride, total cholesterol, VLDL, LDL with no any effect on HDL at the dose of 240 mg/kg/day during 90 days of dosing which may suggests of dyslipidemic effect of Zincovit syrup, in long term usage at higher dose. Increased serum total triglyceride and VLDL even at 60 mg/kg/day further indicates the signal of cardiovascular complications. The significant increase in ALT level in all the three dose treatment groups of Zincovit syrup (15, 60 and 240 mg/kg) generates the signal of its hepatic impairment effect in Wistar rats. This might be due to the presence of lysine as one of the constituent of zincovit syrup. Recently on May 28, 2015, a study conducted by http://www.ehealthme.com/ds/lysine/hepatotoxicity among 458 people reported to have side effects when taking lysine. Among them, 1 people (0.22%) have hepatotoxicity [8]. In the present study lower dose (15 mg/kg) of Zincovit syrup was found to be safer than its higher doses (60 and 240 mg/kg) in Wistar rats. Since toxicity in humans cannot always be entirely extrapolated from animal studies, further clinical evaluation should be performed to precisely define the safe dosage to advice Zincovit syrup as nutritional food supplement in humans.

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