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Research Article

# INFLUENCE OF ZINCOVIT DROP (NUTRITIONAL FOOD SUPPLEMENT) ON IMMUNE SYSTEM IN NORMAL AND CYCLOPHOSPHAMIDE INTOXICATED WISTAR RATS

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**ABSTRACT**

**Objective:** The aim of the present study was to investigate the influence of Zincovit (ZVT) drop (nutritional food supplement) on the immune system in normal healthy and cyclophosphamide (CP) intoxicated Wistar rats.

**Methods:** The study was carried out in healthy and immunotoxic Wistar rats (CP; 20 mg/kg/day) with ZVT drop (a combined formulation of multivitamin-multimineral, flaxseed oil, and lysine) at the dose of 25 and 50 mg/kg/day for 45 days. Hematological and immunological parameters, such as immunoglobulin (Ig)G, IgM, interferon-γ, interleukin-2, and interleukin-4, were assessed. In addition, histopathological examination of the spleen was also performed.

**Results:** There were no significant changes in all the hematological parameters such as total leukocyte, differential leukocyte, total erythrocyte, and total platelets count, hemoglobin amount and also the immunological parameters such as IgG, IgM, interferon-γ, and interleukin-4 level among the treatment groups except interleukin-2. There was a significant increase in interleukin-2 level in the group of animals treated with ZVT drop at the dose of 25 mg/kg ($p<0.001$) and 50 mg/kg ($p<0.05$) in comparison with normal control animals.

**Conclusion:** The present study revealed the immunomodulatory effect of ZVT drop at the dose of 25 and 50 mg/kg/day in normal healthy Wistar rats.

**Keywords:** Zincovit drop, Flax seed oil, Multivitamin-multimineral nutritional food supplement, Cyclophosphamide, Immunomodulatory.

## INTRODUCTION

The immune system is a system of biological structures and processes within an organism that protects against disease. Disorders of the immune system can result in autoimmune diseases, inflammatory diseases and cancer and immunodeficiency [1]. The immune system protects the body against invading pathogens by generating several cells and molecules which eliminate the invaders [2]. Immunomodulators are being used as a supportive adjunct to specific antibiotic therapy in immunodeficient patients [3]. Cyclophosphamide (CP) is an alkylating agent widely used in cancer chemotherapy [4,5]. Its cytotoxic effects are the result of chemically reactive metabolites that alkylate DNA and protein, producing cross-links [6]. The injury of normal tissues is the major limitation of using CP, which gives rise to numerous side effects [7,8]. It has been reported that oxidative stress-mediated disruption of redox balance after CP exposure generates biochemical and physiological disruptions [9,10]. The concept of immunomodulation relates to non-specific activation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells, and lymphocytes, and also to the production of various effector molecules generated by activated cells [11]. Zincovit (ZVT) drop is a combined formulation of vitamins, minerals, lysine, and flaxseed oil. 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. Flaxseed oil is a rich source of the essential fatty acid alpha-linolenic acid, which is a biologic precursor to omega-3 fatty acids. ZVT drop releases a stream of antioxidant benefits [12]. Hence, the aim of the present study was to evaluate the influence of orally administered ZVT drop on the immune level in normal healthy and CP-induced immunotoxic Wistar rats.

## MATERIALS AND METHODS

### Drugs and reagents

ZVT drop was procured from Apex Laboratories Private Ltd., Chennai (India). The enzyme-linked immunosorbent assay (ELISA) kits for immunoglobulin (IgG, IgM, interferon-γ, interleukin-2, and interleukin-4) were obtained from USCN Life Sciences(USA). Sodium chloride and all other chemicals were obtained 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

Fourty two young male Wistar rats (4-6 weeks old) were selected for the study, which were bred locally in the central animal house of Manipal University, Manipal, Karnataka, India. They were housed individually in polypropylene cages containing sterile paddy husk (procured locally) as bedding throughout the study and maintained under standard conditions with temperature (22-24°C), 12 hrs light/12 hrs dark cycle, and relative air humidity 40-60%. The animals were acclimatized to the laboratory conditions for 1 week before the start of the experiment. Rats had continuous access to norm caloric standard rat pellet diet and to tap water. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC/KMC/40/2013), and experiments were conducted according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

## EXPERIMENTAL DESIGN

In the experiment, a total of 42 Wistar rats (18 normal rats, 24 CP intoxicated surviving rats) were used [13]. The rats were divided into 7 groups of 6 rats each. After taking the body weight of each rat, treatment was done orally for 45 days as follows: Group I: Normal

control rats - 2% gum acacia (1 ml/kg/day); Group II: Negative control rats - CP (20 mg/kg/day) + 2% gum acacia (1 ml/kg/day); Group III: Positive control rats - CP (20 mg/kg/day) + 2% gum acacia (1 ml/kg/day) + Levamisole (50 mg/kg/day); Group IV: Test rats - CP (20 mg/kg/day) + ZVT drop (25 mg/kg/day); Group V: Test rats - CP (20 mg/kg/day) + ZVT drop (50 mg/kg/day); Group VI: Test rats - ZVT drop (25 mg/kg/day); Group VII: Test rats - ZVT drop (50 mg/kg/day).

**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 hrs fast. Blood was collected from retro-orbital plexus through the capillary tube. Serum was obtained by centrifugation of blood at 3,000 rpm for 20 minutes at 4°C using a refrigerated centrifuge (MIKRO 22R, Andreas Hettich GmbH & Co. KG, Germany). Thereafter, animals were euthanized by cervical dislocation according to the annexure-6 of euthanasia of laboratory animals in the CPCSEA guidelines for Laboratory Animal Facility.

**Hematological parameters**

About 0.5 ml of blood from each animal was collected in ethylenediamine tetraacetic acid containing vacutainer. Further red blood corpuscles (RBC), white blood corpuscles (WBC), differential leukocytes, platelet count, and amount of hemoglobin were measured with the help of veterinary automatic blood cell counter.

**Immune parameters**

Serum IgG, IgM, interferon-γ, interleukin-2, and interleukin-4 level were measured according to the standard protocol given along with their respective assay kit of USA by using an ELISA reader Bio Tek Instruments ELx800-MS, (USA).

**Qualitative histopathological examination of spleen**

The spleen tissue samples were taken randomly from the 1-2 animals of each group and fixed in 10% phosphate buffered formalin. A small part of the liver was cut and dehydrated in ascending grades of alcohol, defatted in xylene, and embedded in paraffin. 24 hrs after block preparation, 6 µm thick paraffin sections were obtained using microtome and mounted on albumenized glass slides followed by their respective labeling. Tissues were then de-waxed in xylene for 10 minutes and further hydrated through descending grades of alcohol to water. The sections were stained with hematoxylin and eosin. At the end, 2-3 drops of DPX mountant was put on the glass slides and the coverslips were placed gently to avoid drying of tissue and then observed for any morphological changes under a light microscope (Magnus, Olympus Private Ltd., New Delhi, India) ×40. Later the microscopic slides of the spleen cells were photographed.

**Statistical analysis**

Using Statistical Package for the Social Sciences (SPSS version 20.0; SPSS Inc., Chicago, USA), normally distributed data were expressed as mean ± standard deviation and analyzed by one-way analysis of variance followed by *post-hoc* Tukey test. A level for $p \leq 0.05$ was considered to be statistically significant.

## RESULTS

**Effect on hematological and immunological parameters**

There were no significant changes in all the blood parameters such as total leukocyte, differential leukocyte, total erythrocyte, and total platelets count, hemoglobin content and also the immunological parameters such as IgG, IgM, interferon-γ, and interleukin-4 level among the treatment groups except interleukin-2 (Tables 1 and 2). There was a significant increase in Interleukin-2 level in the group of animals treated with ZVT drop at the dose of 25 mg/kg ($p<0.001$) and 50 mg/kg ($p<0.05$) in comparison with normal control animals (Table 2).

**Effect on histopathology of spleen sections**

Histology of spleen of normal control animal displayed normal spleen architecture. The lymphatic nodules were intact, and abundant macrophages were not observed (Fig. 1a). There were no remarkable changes in the spleen of the group of animal treated with only ZVT drop at the dose of 25 and 50 mg/kg/day when compared with normal control but in comparison with CP intoxicated animals, they had intact lymphatic nodules (Fig. 1f and g). In the CP intoxicated negative control (untreated) group, lymphatic nodules were dispersed, and more macrophages were seen (Fig. 1b). These features were very mild in CP + Levamisole, ZVT drop 25 mg/kg/day, and 50 mg/kg/day treatment group (Fig. 1c-e).

## DISCUSSION

Modulation of the immune system by the cytostatic agents is emerging as a major area in pharmacology, especially in cases, where undesired

**Table 1: Effect of ZVT drops on RBC (×10⁶ cells/µl), IgG (µg/ml), IgM (ng/ml), and interferon-γ (pg/ml)**

| Groups (n=6) | RBC | IgG | IgM |
|---|---|---|---|
| I - Normal control (2% gum acacia; 1 ml/kg/day) | 9.22±0.22 | 11.14±1.40 | 12.52±1.12 |
| II - Negative control (CP 20 mg/kg/day+2% gum acacia; 1 ml/kg/day) | 6.18±0.14**[a] | 7.24±1.28**[a] | 8.10±0.02**[a] |
| III - Positive control (CP 20 mg/kg/day+Levamisole 50 mg/kg/day) | 7.16±0.72 | 9.94±0.82 | 10.68±0.01 |
| IV - Test "A" (CP 20 mg/kg/day+ZVT drop 25 mg/kg/day) | 7.02±0.40 | 9.77±0.70 | 10.94±0.03 |
| V - Test "B" (CP 20 mg/kg/day+ZVT drop 50 mg/kg/day) | 7.29±0.67 | 9.28±0.79 | 10.86±0.04 |
| VI - Test "C" (ZVT drop 25 mg/kg/day) | 8.26±0.96 | 10.40±0.86 | 11.81±0.06 |
| VII - Test "D" (ZVT drop 50 mg/kg/day) | 9.76±1.60 | 10.14±0.54 | 11.73±0.03 |

n: Number of rats in each group, RBC: Red blood corpuscles, ZVT: Zincovit; Ig; Immunoglobulin. Values are expressed as mean±standard deviation. **[a]Indicates statistically significant difference compared to normal control group ($p<0.01$)

**Table 2- Effect of ZVT drops on interleukin-2 (pg/ml), interleukin-4 (pg/ml), and interferon-γ (pg/ml)**

| Groups (n=6) | Interleukin-2 | Interleukin-4 | Interferon-γ |
|---|---|---|---|
| I - Normal control (2% gum acacia; 1 ml/kg/day) | 8.72±0.19 | 8.72±0.09 | 8.98±1.36 |
| II - Negative control (CP 20 mg/kg/day+2% gum acacia; 1 ml/kg/day) | 5.75±0.28**[a] | 5.62±0.08**[a] | 5.14±0.10**[a] |
| III - Positive control (CP 20 mg/kg/day+Levamisole 50 mg/kg/day) | 7.00±1.18 | 6.21±1.45 | 7.55±0.21 |
| IV - Test "A" (CP 20 mg/kg/day+ZVT drop 25 mg/kg/day) | 8.06±1.21 | 6.86±0.18 | 8.22±0.53 |
| V - Test "B" (CP 20 mg/kg/day+ZVT drop 50 mg/kg/day) | 7.98±1.15 | 6.90±0.15 | 7.60±0.26 |
| VI - Test "C" (ZVT drop 25 mg/kg/day) | 12.34±0.06***[a] | 8.21±0.14 | 7.62±0.06 |
| VII - Test "D"(ZVT drop 50 mg/kg/day) | 11.42±0.28*[a] | 8.35±0.13 | 7.80±0.12 |

n: Number of rats in each group, RBC: Red blood corpuscles, ZVT: Zincovit. Values are expressed as mean±standard deviation. ***[a]Indicates statistically significant difference compared to normal control group ($p<0.001$), **[a]Indicates statistically significant difference compared to normal control group ($p<0.01$), *[a]Indicates statistically significant difference compared to normal control group ($p<0.05$)

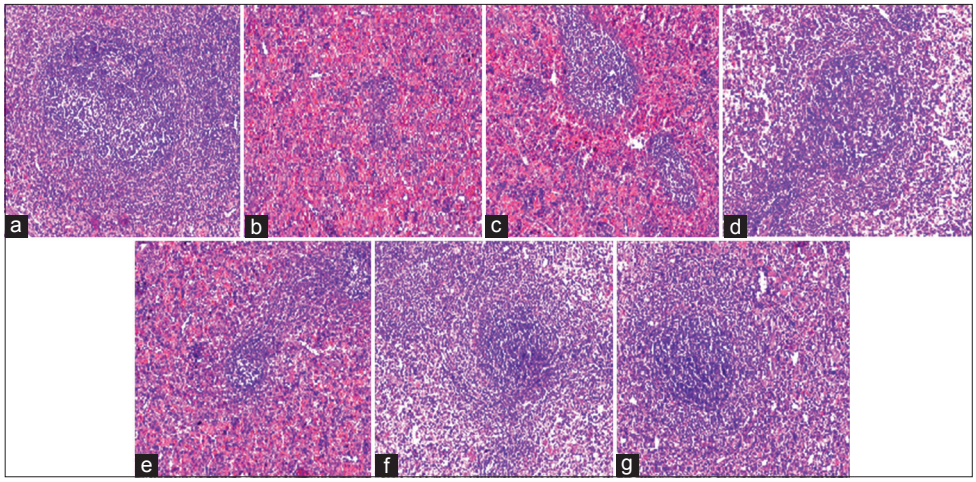


**Fig. 1: (a-g) Microphotographs of hematoxylin and eosin stained sections of liver seen under ×40. (a) Normal control (2% gum acacia; 1 ml/kg/day); (b) Negative control (Cyclophosphamide [CP]; 20 mg/kg/day + 2% gum acacia; 1 ml/kg/day); (c) Positive control (CP; 20 mg/kg/day + Levamisole; 50 mg/kg/day); (d) Test "A" (CP; 20 mg/kg/day + Zincovit [ZVT] drop; 25 mg/kg/day); (e) Test "B" (CP; 20 mg/kg/day + ZVT drop; 50 mg/kg/day); (f) Test "C" (ZVT drop; 25 mg/kg/day); (g) Test "D"(ZVT drop; 50 mg/kg/day)**

immunosuppression is the result of therapy. Cytotoxic drugs such as CP and azathioprine act at various levels on cells involved in defense against foreign invaders [14]. Immunomodulatory agents can alter the immunological responsiveness of an organism by interfering with its regulatory mechanisms. They may selectively activate either cell-mediated or humoral immunity by stimulating either Th1 or Th2 type cell response, respectively. Immunomodulatory agents that are free from side effects and which can be administered for a long duration to obtain a continuous immune activation are highly desirable for the prevention of diseases. Many naturally and chemically derived compound have been discovered with the immunomodulatory activity such as levamisole, glucan, IL-2, interferon, etc., and these are used in combination with cisplatin, adiramycin, 5-flurourcil, etc., against many types of cancer. However, most of them have side effects namely fever, myaligias fatigue, etc [15]. The role of phagocytosis is primary the removal of microorganism and foreign bodies, but also the elimination of dead or injured cells. Phagocytic defects are associated with the varied pathological condition in humans [16]. In the present study, no significant changes were observed in the hematological parameters between the normal and immunotoxic control and the ZVT drop treated groups which suggest that the ZVT drop did not affect either the circulating red cells, or the hematopoiesis that could otherwise have caused a megaloblastic anemia, abnormal differential leukocyte count, platelets count, and hemoglobin. In the present study, ZVT drop has no significant activity on blood cell as well as immune parameters except interleukin-2 in both normal and CP intoxicated group of animals. ZVT drop is found to have mild potent activity on immune level, especially interleukin-2 in normal animals. The ability of ZVT drop to revert the reduced catalase activity buttresses its antioxidant potential. Based on the experimental results reported in the previous study, we concluded that ZVT drop may play an important role in medicine by scavenging free radicals, stimulating activities of antioxidant enzymes subsequently protecting the liver against $CCl_4$-induced damage [12]. We hypothesized that the components (Vitamin C, Vitamin E, zinc, magnesium, and selenium) in combination with other components (lysine and flax seed oil) present in the ZVT drop might be responsible for the reduction of oxidative stress in treatment groups and this might be involved in its immunoprotective role. We did not get any beneficial effect of ZVT drop in terms of boosting immunity except the interleukin-2 level in normal healthy animals. Even the negative impact on immunity was also not found. Therefore, ZVT drop can be used as a nutritional food supplement in mild immunocompromised conditions.

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**REFERENCES**

1. Ukpo G, Ogbonnia S, Ehianeta T, Bashir S. Immunostimulatory and biochemical effects of ethanolic extract of *Mangifera indica* stem bark on dexamethasone-induced immunosuppressed male rats. Int J Pharm Pharm Sci 2013;5(2):569-72.
2. Dashputre NL, Naikwade NS. Preliminary immunomodulatory activity of aqueous and ethanolic leaves extract of *O. basilicum* Linn. in mice. Int J PharmTech Res 2010;2(2):1342-49.
3. Kayser FH, Bienz KA, Eckert J, Zinkernagel RM. Medical Microbiology (eBook). Stuttgart, NY: Thieme; 2005.
4. Baumann F, Preiss R. Cyclophosphamide and related anticancer drugs. J Chromatogr B Biomed Sci Appl 2001;764(1-2):173-92.
5. Fleming RE. An overview of cyclophosphamide and ifosfamide pharmacology. Pharmacotherapy 1997;17:1465-545.
6. Hales BF. Comparison of the mutagenicity and teratogenicity of cyclophosphamide and its active metabolites, 4-hydroxycyclophosphamide, phosphoramide mustard, and acrolein. Cancer Res 1982;42(8):3016-21.
7. Bukowski RM. The need for cytoprotection. Eur J Cancer 1996;32A Suppl 4:S2-4.
8. Fraiser LH, Kanekal S, Kehrer JP. Cyclophosphamide toxicity. Characterising and avoiding the problem. Drugs 1991;42(5):781-95.
9. Das UB, Mallick M, Debnath JM, Ghosh D. Protective effect of ascorbic acid on cyclophosphamide-induced testicular gametogenic and androgenic disorder in male rats. Asian J Androl 2002;4:201-7.
10. Haque R, Bin-Hafeez B, Ahmad I, Parvez S, Pandey S, Raisuddin S. Protective effects of *Emblica officinalis* Gaertn. in cyclophosphamide-treated mice. Hum Exp Toxicol 2001;20(12):643-50.
11. Ghule BV, Yeole PG. *In vitro* and *in vivo* immunomodulatory activities of iridoids fraction from *Barleria prionitis* Linn. J Ethnopharmacol 2012;141(1):424-31.
12. Satyam SM, Bairy KL. Zincovit drop reduces oxidative stress induced by carbon tetrachloride in rats. AJPTT 2015;3(5):469-74.
13. Sahu MS, Sahu RA, Verma A. Immunomodulatory activity of alcoholic extract of *Habenaria intermedia* in mice. IJPPS 2013;5(3):406-9.
14. Kuby J. Immunology. 3rd ed. New York: WH Freeman Company; 1997. p. 436.
15. Ziauddin M, Phansalkar N, Patki P, Diwanay S, Patwardhan B. Studies on the immunomodulatory effects of *Ashwagandha*. J Ethnopharmacol 1996;50(5):69-76.
16. Herberman RB, Pinsky CM. Polyribonucleotides for cancer therapy: Summary and recommendations for further research. J Biol Response Mod 1985;4(6):680-4.