GENOTOXIC EVALUATION OF KOVIR CAPSULES USING MAMMALIAN BONE MARROW CHROMOSOMAL ABERRATION TEST

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The genotoxic potential of KOVIR capsules was investigated in vivo using the mammalian bone marrow chromosomal aberration test. Four groups of mice, seven in each group, were used in the study: Group I served as vehicle control; Group II received the positive control (cyclophosphamide 50 mg/kg; single dose; intraperitoneal route); and Group III to IV were given orally the KOVIR capsules, suspended in distilled water, at a single test dose of 2.16 and 4.32 capsules/kg, respectively. The obtained data disclosed the safety of KOVIR capsules, which did not show substantial genotoxic effects at either dose when compared with the vehicle group.

Keywords: KOVIR, bone marrow, chromosome aberration, mice.

I. INTRODUCTION

Immunology is an ever-evolving field with broad applications in biomedicine. One of the important research directions of immunity is to understand the factors in the regulatory network, controlling the activity of this system. During the functioning of the immune system, many substances play a role in communicating information between cells. These substances have the effect of stimulating or inhibiting the maturation and functional activities of immune cells. Substances are called immunostimulants when they energize the immune response.[1] These substances play a significant role in aiding the treatment of immunodeficiency conditions, chronic infections, or cancer.[2]

Immunostimulators can be derived from natural sources or can be chemically synthesized. KOVIR, a Vietnamese herbal-derived hard capsule, has been developed based on the formula of *Ren Shen Bai Du San* remedy. This polyherbal product consists of 12 herbal ingredients and has been proven to have immunostimulating effects *in vivo*.[3] KOVIR capsules are expected to be a beneficial therapy for various diseases that need to enhance the activities of the immune system.

The broad term “genotoxicant” refers to a chemical that adversely affects genetic components through any of many mechanisms, including mutation, but does not necessarily imply the ability to cause heritable changes. When referring to genotoxicity testing, what is commonly understood is mutagenicity testing.[4] Testing for mutagenicity is the first stage in chemical classification and an essential component of regulatory approval and marketing, whether it is industrial chemicals, pesticides, drugs, dietary supplements, or food additives. Furthermore, mutagenicity testing reduces the genetic or carcinogenic risk to humans since several studies have shown a link between mutagenicity and carcinogenicity.[5]

The present study aimed to examine the genotoxic potential of the KOVIR capsules using the chromosomal aberration test in laboratory mice.

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II. MATERIALS AND METHODS

1. KOVIR capsules

KOVIR capsule was developed based on the *Ren Shen Bai Du San* formulation by the Thai Duong Joint Stock Company. Each 800 mg hard capsule contained 600 mg fine powder of mixed herbal medicines and 200 mg excipient (corn starch, calcium carbonate, aerosil, talc, magnesium stearate, and sodium benzoate). The powder of the herbal mixture was extracted from 12 herbal ingredients (Radix Bupleuri chinensis, Poria, Radix Codonopsis pilosulae, Radix Peucedani, Radix et Rhizoma Glycyrrhizae, Radix Platycodi grandiflori, Rhizoma Ligustici wallichii, Fructus Aurantii, Rhizoma et Radix Notopterygii, Radix Angelicae ubescentis, Rhizoma Zingiberis, Herba Menthae) with water as the extraction solvent.

The predicted human dose of KOVIR was nine capsules per day. Suspension of KOVIR was prepared in distilled water just before use and administered orally.

2. Animals

Adult *Swiss* mice (20-25 g) were purchased from the National Institute of Hygiene and Epidemiology (Vietnam). The animal experiment was performed according to the guideline for the care and use of laboratory animals.[6] The mice were housed in metal cages in groups of 10 animals/cage. Before the experience, all animals were maintained 7 days under the same laboratory conditions of temperature ($22^0C \pm 3^0C$), relative humidity (55% ± 5%), and a 12:12 hours light/dark cycle and received a nutritionally standard diet (NIHE, Vietnam) and tap water *ad libitum*.

3. Experimental Design

The study was conducted from August 2022 to December 2022 at the Department of Pharmacology and Department of Medical Biology and Genetics, Hanoi Medical University.

The mammalian *in vivo* chromosomal aberration test was used to detect structural chromosomal aberrations induced by the test compounds in animal bone marrow cells according to the method described by OECD.[7]

After an acclimation period, mice were randomly divided into four groups (07 mice per group).

- Group 1 – Vehicle control group was administered with distilled water

- Group 2 – The positive control group was administered a single dose of 50 mg/kg b.w cyclophosphamide (CYP) (Baxter Oncology GmbH, Germany).

- Group 3 – Low-dose KOVIR treated with a single dose of 2.16 capsules/kg b.w test product.

- Group 4 – High-dose KOVIR treated with a single dose of 4.32 capsules/kg b.w test product.

Animals were administered oral gavage with the test item or vehicle. Positive control was administered by intraperitoneal route. Colchicine (Gibco® KaryoMAX™ Colcemid™ Solution in PBS 10 µg/mL) at the dose of 4 mg/kg b.w was injected into all the animals intraperitoneally after 12 hours of treatment and 3 hours before sacrifice. Slides for chromosome analysis were arranged and stained as described in previous studies.[8,9,10]

Immediately after the animals were sacrificed, the femurs of mice in all groups were freed from adherent tissues and dissected. A needle with a syringe filled with 37^0C warm hank solution was inserted through the opening at the lower end of the femur bone to collect bone marrow into the prelabelled Falcon tubes. The cell suspension was centrifuged at 1200 rpm for

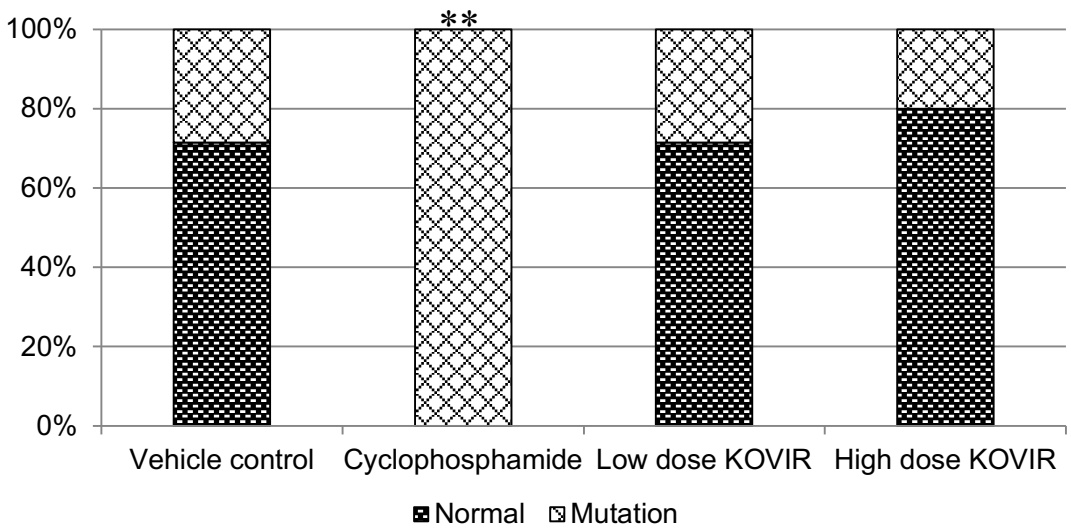
10 minutes and the supernatant was discarded. The pelleted cells were re-suspended in approximately 10 mL KCl hypotonic solution and incubated at 37^0C for 30 minutes. After exposure to the hypotonic solution, the cells were centrifuged at 1200 rpm for 10 minutes at room temperature and the supernatant was discarded. The residual cell pellet was mixed with chilled Carnoy's fixative for the fixation of cells. The cells were centrifuged again at 1200 rpm for 10 minutes at room temperature and the supernatant was discarded. This fixation procedure was repeated two times. After centrifugation, this cell suspension was dropped on a clean chilled slide by hanging drop method and kept for air-drying. Air-dried slides were stained for 15 minutes in 5% Giemsa stain, rinsed in deionized distilled water, and allowed to dry in the dark at room temperature.

All slides were independently coded before microscopic analysis. The slides were scored for gaps, breaks, fragments, deletions, exchanges, and chromosomal disintegrations as structural chromosome aberrations. The chromosomal number mutations were also determined.

4. Statistical Analysis

All the data were expressed in mean ± standard deviation (SD). Data were analyzed using the Chi-square test and Mann-Whitney U test. All statements of significance were based on a probability (P) ≤ 0.05.

III. RESULTS



**p<0.01 as compared with vehicle control (Chi-square test)

Figure 1. The percentage of mice with chromosomal mutations

As presented in Figure 1, the percentage of mice with chromosomal mutations in vehicle control, cyclophosphamide, low-dose KOVIR, and high-dose KOVIR group was 28.57%, 100%, 28.57%, and 20%, respectively. The mutation percentage in both KOVIR groups was not different from the vehicle control group. There was no difference in the mutation percent when compared between the two KOVIR groups at different test doses.

Table 1. Percentage of normal/abnormal chromosome clusters

Groups	Percentage (%) (± SD)	
	Normal clusters	Abnormal clusters
Vehicle control	98.08 ± 3.79	1.92 ± 3.79
Cyclophosphamide	48.07 ± 9.85***	51.93 ± 9.85***
Low dose KOVIR	98.57 ± 2.62	1.43 ± 2.62
High dose KOVIR	98.33 ± 3.73	1.67 ± 3.73

****p<0.001 as compared with vehicle control (Mann-Whitney U test)*

Table 1 summarizes the percentage of normal/abnormal chromosome clusters that were observed in bone marrow cells after treatment with different doses of KOVIR. Intraperitoneal injection of cyclophosphamide at a dose of 50 mg/kg significantly increased the percentage of abnormal chromosome clusters in the bone marrow cells compared with the vehicle control group, and the difference was statistically significant with $p < 0.001$. The percentage of abnormal chromosome clusters in the bone marrow of mice taking KOVIR at doses of 2.16 and 4.32 capsules/kg had no difference compared with the normal control group ($p > 0.05$).

Table 2. The proportion of the number of chromatid aberrations to the total number of chromosome clusters

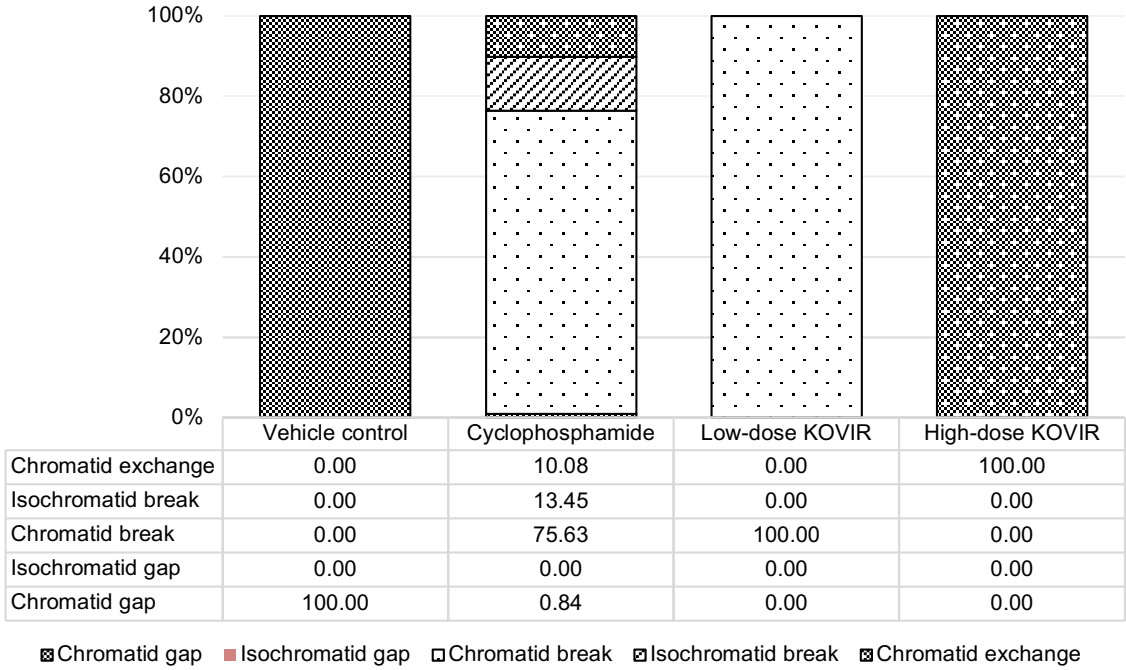
Group	No. of clusters of chromosomes	No. of chromatid aberrations	No. of chromatid aberrations/ No. of clusters of chromosomes
Vehicle control	184	3	0.0163
CYP 50 mg/kg	159	119	0.7484
Low-dose KOVIR	210	2	0.0095
High-dose KOVIR	103	1	0.0097

Observing the data in Table 2, all groups, including vehicle control, had chromatid aberrations, in which the number of chromatid aberrations/the number of chromosome clusters had the highest value in the CYP-intraperitoneal injected mice.

The different types of chromosomal aberrations in metaphase bone marrow cells of mice are shown in Figure 2. Intraperitoneal injection of cyclophosphamide caused various chromosomal aberrations (chromatid gap, chromatid break, iso chromatid break, chromatid exchange). The type of chromosomal aberration that appeared in the low-dose KOVIR group was chromatid break, while in the high-dose KOVIR group, it was chromatid exchange.

Figure 3 showed that the percentage of chromosome clusters with a mutation in the chromosome number with $2n \leq 39$ in the vehicle control group was 0.54%. Intraperitoneal

injection of cyclophosphamide caused mutations in the chromosome number with 2n ≤ 39 and ≥ 41 with a percentage of 0.63% for each type of abnormal number of chromosomes. Only a change in chromosome number with 2n ≤ 39 was encountered in the low-dose KOVIR group with a value of 0.48%. All chromosomal clusters observed in the high-dose KOVIR group had a typical number of chromosomes with 2n = 40.



	Vehicle control	Cyclophosphamide	Low-dose KOVIR	High-dose KOVIR
Chromatid exchange	0.00	10.08	0.00	100.00
Isochromatid break	0.00	13.45	0.00	0.00
Chromatid break	0.00	75.63	100.00	0.00
Isochromatid gap	0.00	0.00	0.00	0.00
Chromatid gap	100.00	0.84	0.00	0.00

Figure 2. The different types of chromosomal aberrations in metaphase bone marrow cells of mice (2n = 40)

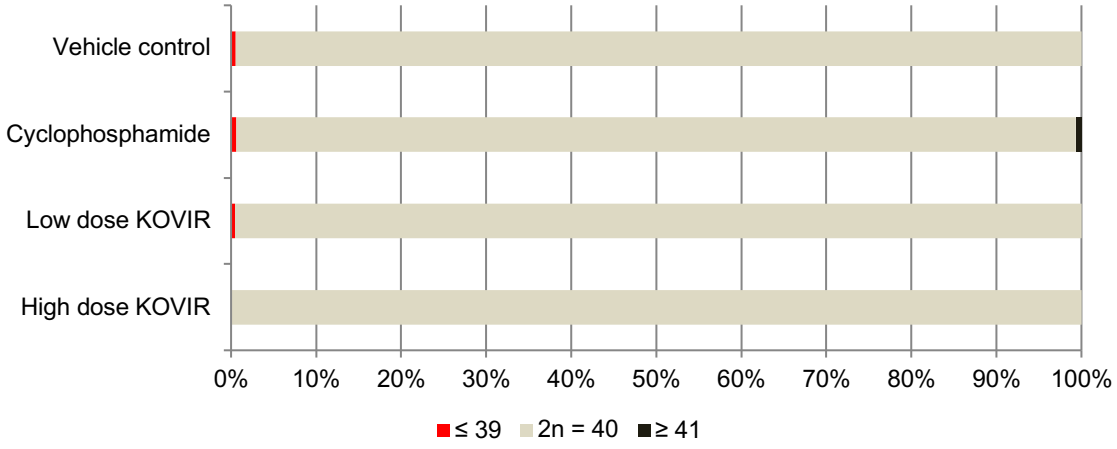


Figure 3. The percentage of different types of chromosome number mutations

IV. DISCUSSIONS

To provide broad coverage of a chemical's mutagenicity and presumably carcinogenicity, information is required on the genotoxic effects of different levels, e.g. the gene, the chromosome, and the cellular apparatus necessary for chromosome segregation. Several experimental procedures, both *in vitro* and *in vivo*, have been designed to assess the effects of chemicals on genetic material, thereby assessing risks to living organisms including humans.[10]

A cytogenetic marker, such as chromosomal aberrations (CAs), is one of the most validated and widely used end-points for the quantification of the biological effects of DNA-damaging agents.[10] CAs are changes in the arrangement of chromosomes due to disruption or alteration of chromosomal material. In general, CA detected in cells is harmful, but some anomalies do not impair cell viability, alternatively, the genetic effects are inherited.[9] The test has been suggested for routine analysis, and the data obtained are considered highly relevant in the human situation.[10]

In our study, the *in vivo* mammalian bone marrow chromosomalaberration test was conducted according to OECD guidelines for the testing of chemicals. Since bone marrow is a highly vascularized tissue and can be easily isolated and processed because of its rapid cell cycle, it has been used as the standard to test for mutagenic potential in various animal models.[8]

In the present investigation, we have compared the genotoxicity of cyclophosphamide and KOVIR capsules. Cyclophosphamide, the positive control chemical, is a well-known alkylating agent and is commonly used as an antineoplastic agent to treat certain malignancies. This compound produces a carbonium ion that reacts with DNA and proteins to induce lethal mutations, generate ROS, and lead to cell damage.[11] Several studies have reported that CYP administered by the intraperitoneal route can cause DNA damage, chromosomal abnormalities, sister chromatid exchange, and a decreased mitotic index.[9] The obtained data are in complete agreement with earlier reported clastogenicity of CYP: 100% of CYP-exposed mice had chromosomal mutations, and 51.93% of observed chromosomal clusters had structural or numerical abnormalities, specifically 119/159 chromosomal clusters with various types of structural aberrations (gaps, breaks, and exchanges) and 2/159 chromosomal clusters with numerical mutations. These results confirmed that even a single dose of cyclophosphamide exerts its genotoxic effect after exposure.

After treatment with KOVIR at both single test doses, the data indicated that no induction of chromosomes or chromatid aberrations in metaphase arrest was found. Neither of the test doses increased the percentage of mice with chromosomal mutations compared with the vehicle control. Abnormal chromosomal clusters representing only < 2% of total chromosome clusters were observed in the bone marrow slides of KOVIR-treated mice, these percentages did not differ from mice just given orally distilled water. Chromosomal aberrations were also present in a small proportion of the total number of chromosome clusters observed, 2/210 and 1/103 for low and high-dose KOVIR, respectively. Unlike cyclophosphamide, which causes various types of chromosome aberrations, only one was observed in low-dose (chromatid break) and high-dose (chromatid exchange) KOVIR

groups. For chromosome number mutations, only the alteration in chromosome number with 2n ≤ 39 was encountered in the low-dose KOVIR group with a percentage of 0.48%, and there are no such mutation in the high-dose KOVIR group.

The above findings have demonstrated that the KOVIR capsule is not a potential bone marrow genotoxic agent when administered to animals. Thus, it is probable that exposure to the KOVIR capsule did not induce a substantial increase in the proportion of structural or number chromosome mutations when compared with the vehicle group.

V. CONCLUSIONS

Based on the evidence generated during the investigation, it can be suggested that the single oral administration of different doses of the KOVIR capsules did not show a significant degree of genotoxicity in mice's bone marrow cells.

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