REPRODUCTIVE/DEVELOPMENTAL TOXICITY SCREENING TEST OF KOVIR CAPSULES IN MICE

Pham Thi Van Anh[1], Dau Thuy Duong[1], Nguyen Huu Thang[2]
Nguyen Thi Huong Lien[2], Nguyen Minh Duc[2] and Mai Phuong Thanh[1,✉]

[1]Hanoi Medical University
[2]Thai Duong Joint Stock Company

The present study was performed to gather information on the effects of Kovir capsules on reproductive and developmental parameters in mice according to the OECD guideline for reproductive/developmental toxicity screening test. The test item was administered orally at dose levels of 1.44 and 2.16 capsules/kg body weight/day to male mice from 2 weeks before mating to the end of the 14-day mating period and females from 2 weeks before mating, during mating, gestation, and until the 13th day of lactation. During the study period, clinical signs, body weight, relative organ weight, reproductive and littering results, necropsy results, and histopathological examination of the testicles were examined. No significant differences existed between the relative organ weights of exposed and unexposed males after the 4-week exposure. Photomicrographs of the testis of treated males did not display marked damage compared with untreated males. There were also no treatment-related effects of Kovir on pregnancy rates, the number of implantations, and the live fetuses of females. These results suggested that Kovir capsules at both test doses did not induce reproductive hazards in mature male and female animals. The effects of Kovir on the mean litter size, postnatal mortality, and appearance of developmental markers in pups were not identified.

Keywords: Kovir, reproductive toxicity, mice.

I. INTRODUCTION

The immune system, a sophisticated and complex network of organs, white blood cells, proteins (antibodies), and chemicals, work together to defend the body against external pathogens, such as microbes (organisms such as bacteria, fungi, and parasites), viruses, cancer cells, and toxins.[1] The state of good health is retained by the regulation of several cellular and humoral factors working in immunoregulatory mechanisms. Understanding the factors that regulate and control the activity of the immune system is one of the important research directions. Substances are called immunostimulants when they energize the immune response.[2] These substances play a significant role in aiding the treatment of immunodeficiency conditions, chronic infections, or cancer.[3] Plant-derived immunostimulants are a promising way to prevent and cure diseases.

Kovir (TD0069), a Vietnamese herbal-derived hard capsule, has been developed based on the formula of *Ren Shen Bai Du San* remedy. This mixed herbal product consists of 12 herbal ingredients and has been proven to have immunostimulating effects *in vivo*.[4] Kovir capsules are expected to be a beneficial therapy for various diseases that require strengthening the immune system.

Corresponding author: Mai Phuong Thanh
Hanoi Medical University
Email: maiphuongthanh@hmu.edu.vn
Received: 17/08/2023
Accepted: 18/09/2023

The requirements for toxicity data in many countries are most stringent for substances intended for direct human use or with expected biological activity, such as pharmaceuticals and pesticides. A crucial aspect of chemical safety assessment (industrial and agricultural chemicals and pharmaceuticals) is the identification of their potential reproductive and developmental toxicity. Evaluation of reproductive toxicity includes probable effects of the chemical on fertility, embryonic and fetal development, prenatal and postnatal development, and parental function.[5,6]

To further characterize the toxicity potential associated with Kovir, we performed this study to examine the potential reproductive and developmental toxicity of Kovir and to determine the relative vulnerability of males and females in *Swiss* mice according to the OECD guideline for reproductive/ developmental toxicity screening test.

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 2-3 capsules, 2-3 times daily. Kovir was suspended in distilled water and administered to the mice by gastric gavage at dose levels of 1.44 capsules or 864 mg polyherbal powder (low dose) and 2.16 capsules or 1296 mg polyherbal powder (high dose) per kg b.w/day (*based on the conversion from an equivalent dose of six capsules/day and nine capsules/day for patients in the clinic, respectively)*. Dose formulations were prepared fresh daily before administration.

2. Animal husbandry

50 male and 100 nulliparous female Swiss mice aged 5-9 weeks were obtained from the National Institute of Hygiene and Epidemiology (Vietnam). Males and females were acquainted with the laboratory for seven days before the start of the experiment. The mice, ten per cage per sex, were kept in an environment maintained at 20~25°C, 40~70% relative humidity, and a 12 h light: 12 h dark cycle. Food and tap water are freely available.

3. Experimental design

The study was conducted from December 2021 to January 2022 at the Department of Pharmacology, Hanoi Medical University.

This study was conducted under the OECD Guidelines for the Testing of Chemicals, Section 4, TG 421 “Reproduction/developmental toxicity screening test”.[7]

After acclimation, for each sex, mice were randomly segregated into three groups, allowing up to 20% difference in mean weight per group. Mice were treated as Table 1.

Table 1. Dosage groups for reproductive/developmental toxicity study in mice

Groups	Males (n = 10)	Females (n = 20)
Control	$1D_0$ group (distilled water)	$1C_0$ group (distilled water)
Low-dose Kovir	**$2D_1$ group (test product)**	$2C_0$ group (distilled water)
	$2D_0$ group (distilled water)	**$2C_1$ group (test product)**
High-dose Kovir	**$3D_1$ group (test product)**	$3C_0$ group (distilled water)
	$3D_0$ group (distilled water)	**$3C_1$ group (test product)**

Each group of mice was exposed to Kovir capsules for two weeks prior to mating. Control animals received only distilled water. Dosage is continued in both sexes during the mating period. The males were given daily doses for a total of at least 28 days until the scheduled sacrifice (one day post-last dose). The daily dosing of the parental females was continued throughout pregnancy and at least until the 13th day postpartum or the day before sacrifice.

4. Mating period

Immediately after the 2-week exposure, males from the control and from each experimental group were caged for 2 weeks with virgin females as trio mating (2 females to 1 male), as shown in Table 2. Half of the mated females in each group were humanely sacrificed on cohabitation day 18. The other half of the females from each group were allowed to deliver and rear their litters.

Table 2. Mating procedure

Groups	Cohabitation
Control	$1D_0$ group (distilled water) + $1C_0$ group (distilled water)
Low-dose Kovir	**$2D_1$ group (test product)** + $2C_0$ group (distilled water)
	$2D_0$ group (distilled water) + **$2C_1$ group (test product)**
High-dose Kovir	**$3D_1$ group (test product)** + $3C_0$ group (distilled water)
	$3D_0$ group (distilled water) + **$3C_1$ group (test product)**

Parameters evaluated

Males of each group were sacrificed at the end of the mating period. The liver, kidneys, spleen, testes, epididymides, seminal vesicles, prostate, glans penis, bulbourethral (Cowper's) gland, and levator ani plus bulbocavernosus muscle complex were weighed. During necropsies, a complete list of organs and tissues was weighed separately to calculate the organ weights per 10 g body weight (relative organ weight). The testis from 3 males in each group were preserved in 10% neutral-buffered formalin and slides prepared for histopathological examination. Sections stained with hematoxylin and eosin were examined under a light microscope. The images were photographed digitally at 20x.

50% of females sacrificed on cohabitation day 18 were examined for the number of

corpus luteum, the number of implantations, the number of live fetuses, and the number of early and late post-implantation deaths. Post-implantation deaths were classified as early, if the embryo had died and had been resorbed, or late, if the dead embryo was at a stage beyond the onset of organogenesis.

50% of females allowed to deliver were examined for the litter size, the number of stillbirths, live births, and the presence of gross anomalies. Live pups were sexed and weighed within 24 hours of parturition (day 0 or 1 post-partum) and on days 4 and 13 post-partum.

5. Statistical analysis

The Chi-square test and Student's t-test were the statistical tests applied using Microsoft Excel version 2010. A p-value less than 0.05 was regarded as significant.

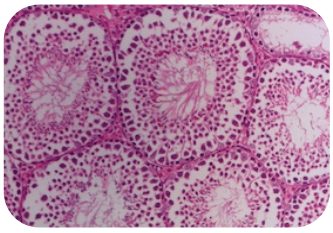
III. RESULTS

1. Effects of Kovir capsules on male mice

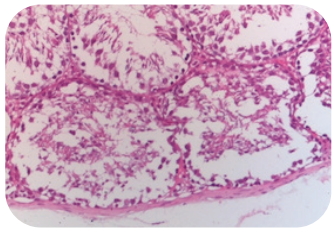
The mean weights of liver, kidney, spleen, and reproductive organs of male mice following 4-week exposure are shown in Table 3. There were no significant difference between the relative organ weights of exposed and unexposed males after the end of the 4-week exposure.

Table 3. Effects of Kovir on the relative organ weight

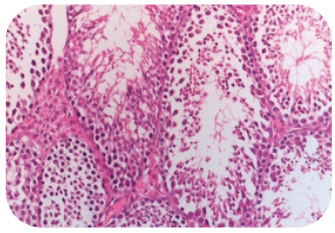
Relative weight (± SD) (mg/10g bw)	Control	Low-dose Kovir		High-dose Kovir	
	Received vehicle	Received Kovir	Received vehicle	Received Kovir	Received vehicle
Liver	575.7 ± 92.5	548.8 ± 53.8	578.8 ± 69.4	510.2 ± 47.2	631.4 ± 106.3
Kidneys	146.2 ± 17.7	135.9 ± 20.4	132.9 ± 15.0	138.5 ± 29.9	135.9 ± 9.5
Spleen	51.0 ± 14.1	47.5 ± 10.9	53.9 ± 17.7	47.3 ± 14.2	52.5 ± 10.6
Glans penis	8.8 ± 0.9	7.6 ± 2.0	8.8 ± 1.6	8.0 ± 2.7	8.6 ± 1.9
Testes	78.7 ± 7.7	77.2 ± 7.9	79.3 ± 15.2	79.9 ± 9.8	72.5 ± 14.3
Epididymides	26.1 ± 4.4	23.3 ± 5.1	22.2 ± 5.1	21.9 ± 5.1	21.3 ± 4.4
Seminal vesicles	13.2 ± 4.3	12.4 ± 3.0	17.2 ± 5.7	16.9 ± 5.1	17.7 ± 5.6
Prostate	4.7 ± 1.1	5.0 ± 1.6	4.7 ± 1.1	5.8 ± 1.5	4.8 ± 1.5
Cowper's glands	5.0 ± 1.2	4.5 ± 1.3	6.1 ± 1.4	6.0 ± 1.6	6.1 ± 1.1
Levator ani	20.6 ± 3.4	22.1 ± 4.4	25.3 ± 6.8	24.4 ± 5.0	23.7 ± 6.9



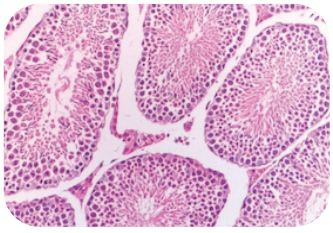
A) Normal seminiferous tubule



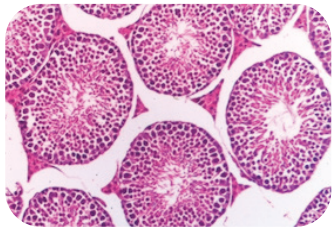
B) Mild edema and degeneration of seminiferous tubules



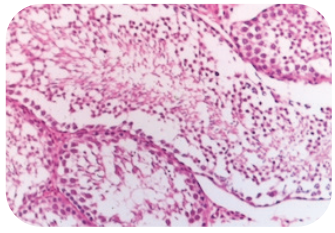
C) Mild degeneration of seminiferous tubules



D) Normal seminiferous tubule

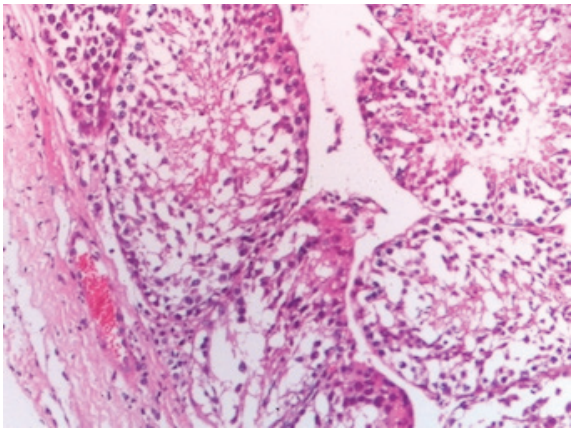


E) Normal seminiferous tubule

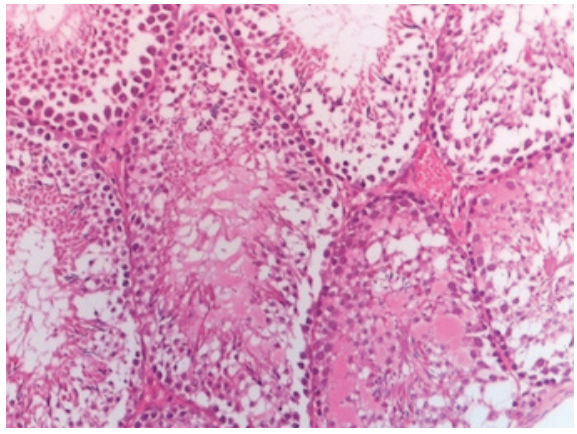


F) Moderate degeneration of seminiferous tubules

Figure 1. Selected microphotographs of testis of animals received distilled water, including mice in $1D_0$ group (A, B), $2D_0$ group (C, D), $3D_0$ group (E, F) (HE, 20x)

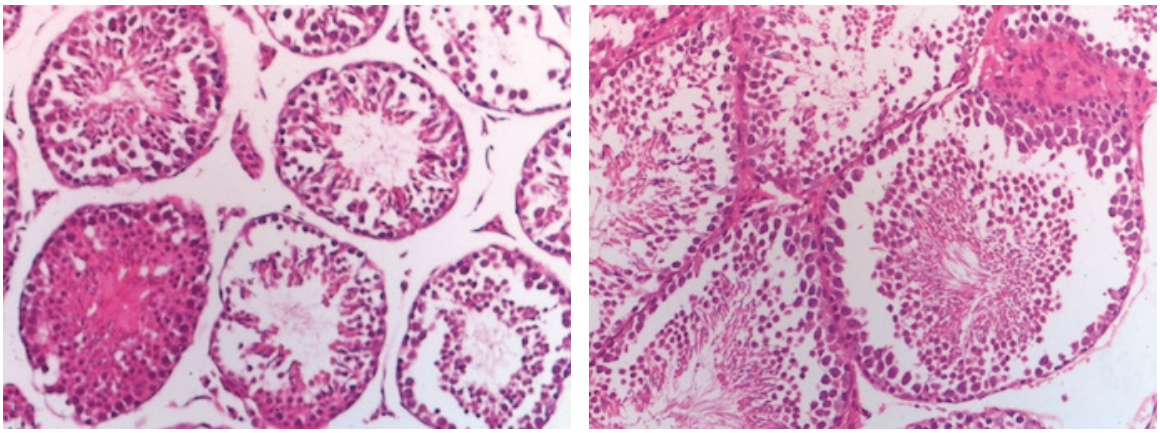


Few degenerative changes in seminiferous tubules



Few necrotic and degenerative changes in seminiferous tubules

Figure 2. Selected microphotographs of testis of low-dose Kovir mice ($2D_1$ – received Kovir at the dose of 1.44 capsules/kg) (HE, 20x)



Few mild degenerative changes in seminiferous tubules

Mild degeneration of seminiferous tubules

Figure 3. Selected microphotographs of testis of high-dose Kovir mice ($3D_1$ – received Kovir at the dose of 2.16 capsules/kg) (HE, 20x)

2. Effects of Kovir capsules on female mice

Table 4. Effect of Kovir on the percent of pregnant females and the number of implantations and live fetuses of 50% of female mice sacrificed on cohabitation day 18

Groups	Cohabitation	Pregnancy rate (%)	No. of implantations	No. of live fetuses	No. of dead fetuses
Control	"both-parents" non-treated	12.5	10	10	0
Low-dose Kovir	"father-only" treated	5.0	8	8	0
	"mother-only" treated	0	0	0	0
High-dose Kovir	"father-only" treated	5.0	12	12	0
	"mother-only" treated	5.6	10	10	0

The results regarding the pregnancy rate of Kovir-exposed and control females and the number of implantations and live fetuses of Kovir-exposed and unexposed females are shown in Table 4. There were no statistically significant effectof Kovir on the frequency of pregnant females ($p > 0.05$). The post-implantation deaths were not noted in all groups.

None of the females allowed to deliver was pregnant. Therefore, the results of mean litter size, postnatal mortality, and appearance of developmental markers in pups of exposed and unexposed Kovir females were not determined.

Table 5. Effect of Kovir on the number of corpus luteum

Groups		No. of corpus luteum (± SD)
Control	received distilled water	5.69 ± 1.58
Low-dose Kovir	received distilled water	6.06 ± 1.75
	received test product	6.30 ± 1.92
High-dose Kovir	received distilled water	5.83 ± 1.65
	received test product	5.50 ± 1.47

Table 5 presents the number of corpus luteum of female mice. There were no significant differences in the mean number of corpus luteum between Kovir-unexposed and exposed female groups (p > 0,05).

III. DISCUSSIONS

Although only 2 - 4% of congenital developmental defects in children are caused by known chemical or physical exposure during pregnancy or even before, the aftereffects of such exposure can be miserable.[5] Harmful effects may exhibit pre- or postnatally, such as developmental and growth retardation, malformations, functional defects, and even death.[8] Since such adverse effects on human development are exclusively preventable if the toxic potential of a substance is known, stringent testing is warranted. The assessment of reproductive toxicity includes adverse effects of substances at all stages of the reproductive cycle, including maternal and fetal fertility, and the developing organism, when evaluating developmental toxicity focus on the later stage.[9]

To date, regulatory reproductive and developmental safety testing of chemicals and drugs has been conducted primarily using animal models and is usually performed in rodents (mice, rats, and rabbits). In general, supervisory safety testing for industrial, agricultural, and other chemicals is typically performed according to the Organisation for Economic Co-operation and Development (OECD) test guidelines. Developmental and reproductive toxicity examinations initiate with screening tests by test guideline 421/422, where exposure begins before mating and investigates effects on the parental and first-generation immediately after delivery.[5]

In the present study, we conducted a reproductive/development toxicity screening test for the Kovir capsules following OECD test guideline 421. The toxicity was investigated in both experimental animal sex.

Male mice were exposed to Kovir for four weeks, including two weeks before mating and 2-week cohabitation. The data in Table 3 showed the mean weight of the liver, kidney, spleen, and reproductive organs (glan penis, testes, epididymis, seminal vesicles, prostate, Cowper gland, and levator muscle) was no difference between Kovir treated and untreated males during the 4-week study period. Microscopic examination of the testes also did not show lesions arising after four weeks of daily intake of Kovir in experimental animals. As above results, reproductive toxicity of Kovir was not observed in mature male mice following 4-week exposure.

The females were given daily doses for at least 28 days until the scheduled sacrifice (one day post-last dose). The daily dosing of the

parental females was continued throughout pregnancy and at least until the 13th day postpartum or the day before sacrifice. Half of the mated females in each group were humanely sacrificed on cohabitation day 18. The other half of the females from each group were allowed to deliver and rear their litters. The data in Tables 4 and 5 show that the harmful impacts of Kovir on pregnancy rates, the number of implantations, and live fetuses were not remarked. In addition, the post-implantation deaths were not noted. There was also no difference in corpus luteum counts when comparing female mice given orally with or without the test product. Based on these outcomes, the reproductive toxicity of Kovir was not indicated in mature female mice. The lack of information on Kovir's impacts on the first-generation immediately after delivery is a limitation of this study. None of the females allowed to deliver became pregnant. Therefore, the results of mean litter size, postnatal mortality, and appearance of developmental markers in pups of exposed and unexposed Kovir females were not determined. Further studies in which the female-to-male ratio was estimated to increase pregnancy and childbirth rates in female mice are needed, which will provide more information on the effects of Kovir on the development of the offspring immediately after birth.

IV. CONCLUSIONS

In summary, the reproductive/developmental toxicity screening test of Kovir at the dose of 1.44 capsules/kg/day and 2.16 capsules/kg/day in mice showed that treatment with Kovir capsules did not induce reproductive hazards in both mature male and female animals. The effects of Kovir on the mean litter size, postnatal mortality, and appearance of developmental markers in pups were not identified.

REFERENCES

1. Marshall JS, Warrington R, Watson W, Kim HL. An introduction to immunology and immunopathology. *Allergy Asthma Clin Immunol*. 2018; 14(Suppl 2): 49. doi: 10.1186/s13223-018-0278-1.

2. Jain P, Darji P, Thakur BS, et al. Immunostimulants: Concepts, Types and Functions. *Asian Journal of Dental and Health Sciences*. 2022; 2:26-34.

3. Mitra S, Paul S, Roy S, Sutradhar H, Bin Emran T, et al. Exploring the Immune-Boosting Functions of Vitamins and Minerals as Nutritional Food Bioactive Compounds: A Comprehensive Review. *Molecules.* 2022; 27(2): 555. doi: 10.3390/molecules27020555.

4. Dinh Thi Thu Hang. Evaluation of toxicity and immunostimulating effects of Kovir capsules on experimental animals. Resident Doctor's Thesis, Hanoi Medical University. 2017. [Vietnamese].

5. Saghir SA, Dorato MA. Reproductive and developmental toxicity testing: Examination of the extended one-generation reproductive toxicity study guideline. *Regul Toxicol Pharmacol*. 2016; 79: 110-117.

6. Hareng L, Pellizzer C, Bremer S, Schwarz M, Hartung T. The integrated project ReProTect: a novel approach in reproductive toxicity hazard assessment. *Reprod Toxicol.* 2005 Sep-Oct; 20(3): 441-52. doi: 10.1016/j.reprotox.2005.04.003. PMID: 15899576.

7. OECD. Test No. 421: Reproduction/Developmental Toxicity Screening Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. 2016. doi: 10.1787/9789264264380-en.

8. Nikolaidis E (2017). Relevance of animal testing and sensitivity of end points in reproductive and developmental toxicity. In:

Reproductive and Developmental Toxicology (ed. R.C. Gupta), Cambridge, MA: Academic Press, 211-224.

9. United Nations Economic Commission for Europe (UNECE). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), ST/SG/AC.10/ 30/Rev.10. 2023. https://unece.org/sites/default/files/2023-07/GHS%20Rev10e.pdf.