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The Study of the Specific Toxicity of "Apiprost" Capsules

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Abstract

Introduction. Chronic prostatitis occurs in 35-45% of cases of urological pathology. In recent years there is a tendency to chronic prostatitis increase among young working-age population. This determines the medical and social importance of the problem of improving the effectiveness of treatment of chronic prostatitis. The development of drugs based on bee products and their standardized substances that have a wide range of the pharmacological activity (antimicrobial, antiviral, anti-inflammatory, immunostimulatory, etc.) is of interest for the treatment of prostatitis. One of these drugs is "Apiprost" capsules developed on the basis of apiculture products – bee pollen (BP) and the propolis phenolic hydrophobic drug (PPHD). In preclinical studies "Apiprost" capsules exhibited the marked anti-inflammatory and prostate protective action. The aim of this work was to study some types of specific toxicity of "Apiprost" capsules, namely the presence of immunotoxic, mutagenic action and the ability to cumulate.

Materials and Methods. The research was performed according to the guidelines on the preclinical studies of drugs.

Results and Discussion. It has been found that "Apiprost" capsules do not reveal the cumulative properties, do not have the mutagenic effect on somatic cells of rats and mice, and do not cause genetic changes in the germ cells of the parent species as evidenced by the absence of death of the first generation offspring at the embryonic stages of development.

Conclusions. "Apiprost" capsules in the dose of 100 do not affect the course of the delayed-type hypersensitivity reaction, do not change the number of plasmocytes in the spleen and the titer of hemagglutinins in the blood serum of immunized mice. The increase in the dose to 500 mg / kg has the immunostimulatory effect on the cellular and humoral immunity of animals.

Key words: toxicological studies, mutagenicity, immunotoxicity, cumulation, propolis phenolic hydrophobic drug, bee pollen, "Apiprost" capsules.

INTRODUCTION

Chronic prostatitis is a disease that is rather common and insufficiently studied, and it is difficult to treat. It affects mostly young and middle-aged men, i.e. the most sexually active, and is often complicated by the copulative and generative dysfunction [1, 2]. The available literature data on the epidemiology of the disease show that chronic prostatitis accounts for up to 35% of all visits to doctors concerning urological problems among the male population of the working age [3, 4, 1]. This determines the medical and social importance of the problem of improving the effectiveness of diagnosis and treatment of chronic prostatitis.

In the treatment of patients with chronic prostatitis it is crucial to improve the blood circulation in the prostate gland, and increase the immunological reactivity along with elimination of the inflammatory process. In this regard, the use of drugs based on bee products and their standardized substances that have a wide range of the pharmacological activity (antimicrobial, antiviral, anti-inflammatory, immunostimulatory, etc.) is of interest for the treatment of prostatitis [5].

One of these drugs is "Apiprost" capsules developed on the basis of apiculture products – bee pollen (BP) and the propolis phenolic hydrophobic drug (PPHD) [6, 7, 8]. The presence of these components in the drug provides a wider range of the pharmacological activity of "Apiprost" capsules compared to monocomponent drugs. In addition, the presence of the sufficient domestic raw material base for manufacturing the product proposed helps to reduce its cost and makes it economically profitable to introduce this drug into industrial production.

In preclinical studies "Apiprost" capsules exhibited the marked anti-inflammatory and prostate protective action [9]. The therapeutic effect of PPHD is due to a significant amount of phenolic compounds, and BP provides various biologically active components (amino acids, lipids, enzymes, coenzymes, all known vitamins, phenolic compounds, carbohydrates, macro- and microelements, phytohormones, etc.) [9, 5, 8]. However, an integral part of preclinical studies is the confirmation of the safety of new drugs [10].

In this regard, the aim of this work was to study some types of specific toxicity of "Apiprost" capsules, namely the presence of immunotoxic, mutagenic action and the ability to cumulate.

MATERIALS AND METHODS.

The experiments were conducted in accordance with "Directive of the EU Council on the protection of animals used for scientific purposes" [11], and approved by the Bioethics Commission of the National University of Pharmacy (NUPh). Animal care and manipulations with animals were performed according to standard operating procedures of the Central Research Laboratory of the NUPh.

The *mutagenic action* of "Apiprost" capsules was studied according to the guidelines on assessment of the mutagenic properties of new drugs [12, 13]. The study of mutagenic properties of "Apiprost" capsules included a number of methods that allowed investigating all types of genetic changes at the somatic and generative cellular level. The effect of the drug on somatic cells was studied by the metaphase chromosome aberration (CA) assay in the bone marrow cells with a single administration to male mice weighing 22-26 g and under conditions of subacute experiment in male rats weighing 180-200 g.

Under the condition of a single administration male mice were randomized into 3 groups of 6 animals each: intact control (IC), negative control (NC, animals received a carrier – distilled water), animals receiving the test sample (TS) in a single intragastric dose of 1000 mg/kg. Animals were removed from the experiment in 6, 24, 48 h after the last administration of the drug (2 h before the solution of colchicine in the dose of 2.5 mg/kg was injected intraperitoneally), Hanks' solution was used to wash out the bone marrow from both thighs at 37°C; using standard methods the cells were treated, fixed, and stained with azur-eosin. The analysis was performed using the immersion microscope lens with magnification of 10×90; 100 metaphases per animal were analyzed according to the generally accepted criteria [12**Error! Bookmark not defined.**, 13].

In the subacute experiment, the drug was given to male rats in the dose of 100 mg/kg for 1 month. Animals were removed from the experiment in 24 h after the last administration of the drug (2 hours before the solution of colchicine was injected intraperitoneally). The bone marrow suspension and preparation of

samples for microscopic examination were performed by the abovementioned methods [12,13].

The mutagenic properties of "Apiprost" capsules were also studied using the dominant lethal (DL) test. Dominant mutations are genetic changes in the germ cells of the parent individuals that lead to the death of the first generation offspring at the embryonic stages of development. The mutagenic effect is manifested in the increase of embryonic mortality of the first generation offspring before and after implantation [12]. Chromosomal mutations have the main contribution to the induced dominant lethality, while genomic (aberration in the number of chromosomes) and gene mutations contribute to a lesser extent. The mutagenic effect is in the increase of embryonic mortality of the first generation offspring before and after implantation. "Apiprost" capsules were administered in the doses of 100 and 1000 mg/kg to male rats for 2.5 months. Then 3 virginile females were placed to males for 1 week. The first day of pregnancy was considered the day of registration of spermatozoids in vaginal smears. Females were removed from the experiment on the 19th day of pregnancy. The following indicators were recorded: the number of yellow bodies, places of implantation, the number of live feti per one female, the fetal weight and size. Based on the data obtained the frequency of dominant lethal mutations, the total embryonic mortality, pre-implantation and post-implantation fetal death were calculated in percent by conventional methods [12, 13].

The immunotoxic properties of "Apiprost" capsules were studied in accordance with the guidelines [14].The study included the assessment of the effect of "Apiprost" capsules on T- and B-cell components of the immune system.

The effect of TS on the cellular immune response was studied in the delayed-type hypersensitivity (DTH) test by the method of K.P. Kitamura [14, 15]. Testing was performed on white outbred mice weighing 18.0-22.0 g. Mice were randomized into 3 groups: immunized control; animals received TS in the doses of 100 and 500 mg/kg intragastrically one hour before immunization and then within the experiment (5 days). Animals were immunized with a single subcutaneous injection of 1% suspension of freshly washed sheep red blood cells (SRBC) in the dose of 0.5 ml per 20 g to the interscapular region. On day 6 the final dose of antigen (10% suspension of SRBC in the dose of 0.02 ml/animal) was administered under aponeurosis of the hind limb, and a similar volume of Hanks solution in the contralateral limb. In 24 h animals were removed from the experiment. The intensity of the local inflammatory reaction was assessed by the difference in the weight of the limbs with the calculation of the response index (RI) by the formula:

$$RI = \frac{\text{weight of the experimental paw} - \text{weight of the control paw}}{\text{weight of the control paw}} \times 100\%$$

The effect of "Apiprost" capsules on the status of the humoral immune response was determined by the number of plasmocytes in the spleen, hemagglutinin titers (HG) in the serum of immunized animals. Mice were randomized into 3 groups of 10 animals each: immunized control; animals received TS in the conditionally therapeutic dose of 100 mg/kg and the dose increased by 5 times – 500 mg/kg, respectively, one hour before immunization and then during the whole period of immunization. Animals were immunized with a single intraperitoneal injection of 3% suspension of SRBC in the dose of 0.2 ml per 20 g. On day 5 the number of plasmocytes was determined by the method of local hemolysis in gel [14, 16], and HG titers was determined by the serial dilution method [14, 17].

The cumulative properties of "Apiprost" capsules were determined by the method of Lim et al. [18, 19]. The experiment was performed on 12 white outbred rats of both sexes weighing 175-195 g. According to the method TS was injected intragastrically to rats for 28 days in increasing doses of 0.1; 0.15; 0.22; 0.34; 0.5; 0.75; 1.12 of the maximum dose injected to animals when studying acute toxicity – 15000 mg/kg. The dose was increased every 4 days taking into account the dynamics of the animal body weight. At the same time, the days of death of animals and the total dose of the TS injected were considered [18, 19].

All actual material was processed by the methods of variation statistics. The data obtained are presented as an average value and its standard error. The intergroup comparison was performed by the nonparametric methods of analysis – Mann-Whitney test and χ^2). Differences were considered to be statistically significant at $p<0.05$. To obtain statistical conclusions the Statistica standard software package (version 6.0) was used [20].

RESULTS AND DISCUSSION.

The effect of "Apiprost" capsules on formation of delayed-type reactions was studied in the DTH test. According to the data obtained (Table 1) "Apiprost" capsules in the conditionally therapeutic dose of 100 mg/kg did not affect the functional activity of T-cells and, therefore, development of the immune response to the introduction of the SRBC antigen. The increase in the dose of TS under study to 500 mg/kg led to a statistically significant increase in the response index by 1.8 times. It indicates the stimulation of the cellular immune response to the introduction of antigen.

The effect of TS on the humoral immunity was assessed by the number of plasmocytes in the spleen and HG titers in the blood serum of mice with the normal immune status [14]. The results are presented in Table 2.

Table 1. **The effect of "Apiprost" capsules in intragastric administration on the course of the DTH reaction in mice**

Groups of animals	n	Dose, mg/kg	Response index
Immunized control (SRBC)	16	–	6.53±0.65
"Apiprost" capsules + SRBC	10	100	7.76±0.62
"Apiprost" capsules + SRBC	9	500	11.70±1.16*

Notes:
1. * – statistically significant differences compared to the immunized control group, Mann-Whitney test;
2. n – the number of animals in each group.

Table 2. The number of plasmocytes in the spleen of white outbred mice injected intragastrically with "Apiprost" capsules, n=9

Groups of animals	Dose, mg/kg	HG titers, Log_2	The number of plasmocytes per a spleen
Immunized control (SRBC)	–	8.33±0.85	7076±1514
"Apiprost" capsules + SRBC	100	8.78±0.40	11449±2573
"Apiprost" capsules + SRBC	500	9.89±0.56	16364±4021*

Notes:
1. * – statistically significant differences compared to the immunized control group, Mann-Whitney test;
2. n – the number of animals in each group.

According to the data obtained the intragastric administration of "Apiprost" capsules in the conditionally therapeutic dose of 100 mg/kg caused an unreliable increase in the number of plasmocytes in the spleen of mice. The use of the drug in the highest dose – 500 mg/kg, as in the previous experiment, led to a significant stimulation of the immune response of the experimental animals to the introduction of a standard antigen of SRBC.

Determination of antibody titers in the blood serum by the agglutination reaction showed that "Apiprost" capsules in the conditionally therapeutic dose did not affect the number of HG – titers of the indicator corresponded to the values of the immunized control (Table 2). The increase in the dose by 5 times led to an insignificant increase in the level of circulating antibodies in the blood serum of the experimental animals. There were no statistically significant differences between the immunized control and experimental groups (Table 2).

Determination of cumulative properties of "Apiprost" capsules by the method of Lim et al. showed the absence of the ability of TS to cumulation. During the whole experiment, the external signs of poisoning and death of animals were not observed. The animals were calm, neat, well-fed, they had all reactions to external stimuli (noise, pain, bright light), the mucous membrane of the eyes, nose and genitals were normal. The absence of animal death did not allow us to calculate the cumulation coefficient. The results of the experiment are given in Table 3.

Table 3. Determination of the cumulative effect of "Apiprost" capsules by the method of Lim at al., dynamics of death in rats depending on the total dose

Days of the experiment	Daily dose		Total dose		Death of animals
	in parts of the dose taken as LD_{50}	in mg/kg	in parts of the dose taken as LD_{50}	in mg/kg	
1	0.1	1500	0.1	1500	–
2			0.2	3000	–
3			0.3	4500	–
4			0.4	6000	–
5	0.15	2250	0.55	8250	–
6			0.70	10500	–
7			0.85	12750	–
8			1.00	15000	–
9	0.22	3300	1.22	18300	–
10			1.44	21600	–
11			1.66	24900	–
12			1.88	28200	–
13	0.34	5100	2.22	33300	–
14			2.56	38400	–
15			2.90	43500	–
16			3.24	48600	–
17	0.5	7500	3.74	56100	–
18			4.24	63600	–
19			4.74	71100	–
20			5.24	78600	–
21	0.75	11250	5.99	89850	–
22			6.74	101100	–
23			7.49	112350	–
24			8.24	123600	–
25	1.12	16800	9.36	140400	–
26			10.40	157200	–
27			11.60	174000	–
28			12.72	190800	–

The possible mutagenic properties of "Apiprost" capsules were studied under conditions of acute and subacute administration. The results of assessment of the potential mutagenic properties TS in the short-term introduction are given in Table 4. It was found that in 6 h after a single administration of "Apiprost" drug in the dose of 1000 mg/kg to mice the level of frequency of CA slightly increased mainly due to the increase in the number of fragments (Table 4) as evidenced by the increase in the number of chromatic agglutination associated with the effect of the drug on the integrity of cell membranes. However, in 24 and 48 h the number of CA in mice decreased to the level of control. The increase in the frequency of CA does not exceed the population oscillations of this indicator, and therefore, it cannot be considered significant. The mitotic activity in animals of experimental groups remained at the level of the intact control (Table 4).

Under conditions of the subacute experiment when introducing "Apiprost" capsules to male rats for 1 month no significant dysfunctions of indicators indicating the chromosome damage were observed in the bone marrow cells (Table 5).

Table 5

Therefore, based on the data obtained it can be concluded that there is no mutagenic effect of the drug on the somatic cells of rats and mice.

When studying "Apiprost" capsules in the DL test it was found that the drug was not able to cause genetic disorders in the germ cells of the experimental animals (Table 6).

The percentage of all types of fetal death did not exceed the control values. The embryos obtained from the females injected with "Apiprost" capsules did not differ from the embryos of female intact rats either in size or weight. The absence of an increase in the frequency of DL with the introduction of TS allows suggesting the absence of mutagenic properties of "Apiprost" capsules.

Therefore, "Apiprost" capsules do not reveal the mutagenic properties in the dominant lethal test when introduced in the dose of 100 and 1000 mg/kg within 2.5 months.

Table 4. The frequency of chromosome aberrations in the bone marrow cells of mice when introducing "Apiprost" capsules in the dose of 1000 mg/kg, n=5

Indicators	Groups of animals			
	Intact control	6 h after drug administration	24 h after drug administration	48 h after drug administration
Fragments, %	0.9±0.42	1.4±0.42*	1.2±0.27	0.8±0.45
Chromosome bridges, %	2.5±1.12	2.5±0.61	2.0±0.79	2.3±0.84
Chromatid bridges, %	1.3±0.57	1.6±0.42	1.3±0.27	1.3±0.57
The number of proper aberrations, %	4.7±1.15	5.5±0.79	4.5±1.0	4.4±0.96
Chromatic agglutination, %	1.6±0.42	2.0±0.87	1.8±0.84	1.5±0.61
Chromosome lagging, %	0.4±0.42	0.7±0.57	0.6±0.42	0.5±0.35
The total number of dysfunctions, %	6.7±1.72	8.2±1.35*	6.9±1.92	6.4±1.24
Mitotic index	1.7±0.47	1.8±0.31	1.6±0.21	1.7±0.48

Notes:
1. * – statistically significant differences compared to the intact control group, Mann-Whitney test;
2. n – the number of animals in each group;
3. the number of cells analyzed – 1000.

Table 5; The frequency of chromosome aberrations in the bone marrow cells of male rats when introducing "Apiprost" capsules, n=6

Indicators	Groups of animals		
	Control	"Apiprost" capsules	
		100 mg/kg	1000 mg/kg
Fragments, %	0.6±0.88	0.8±0.41	0.8±0.52
Chromosome bridges, %	0.9±0.58	0.8±0.42	0.8±0.82
Chromatid bridges, %	0.6±0.20	0.8±0.42	0.9±0.86
The number of proper aberrations, %	2.1±0.58	2.3±0.65	2.6±0.86
Chromatic agglutination, %	0.4±0.38	0.5±0.45	0.5±0.45
Chromosome lagging, %	0.3±0.27	0.4±0.20	0.4±0.38
The total number of dysfunctions, %	2.8±0.88	3.3±0.76	3.5±0.84
Mitotic index	1.5±0.18	1.6±0.18	1.6±0.10

Notes:
1. n – the number of animals in each group;
2. the number of cells analyzed – 1200.

Table 6. The frequency of dominant lethal mutations in rats when administrating "Apiprost" capsules

Indicators	Groups of animals		
	Control	"Apiprost" capsules	
		1000 mg/kg	100 mg/kg
The number of females	14	18	20
The number of yellow bodies	9.8±1.48	10.1±2.1	9.0±1.43
The places of implantation	8.4±1.99	8.8±1.50	8.4±1.54
The total embryonic mortality, %	15.1 (0÷38.0)	12.9 (0÷31.3)	9.0 (0÷25.0)
Pre-implantation fetal death, %	6.9 (0÷25.0)	11.2 (0÷22.5)	7.0 (0÷25.0)
Post-implantation fetal death, %	9.0 (0÷27.3)	1.9 (0÷12.5)	2.1 (0÷14.9)
The number of live feti per one female	8.36±1.95	8.7±1.53	8.2±1.68
The frequency of dominant lethal mutations	–	0	0
The fetal weight	2.4±160	2.6±1.35	2.6±1.23
The fetal size	3.1±0.26	3.2±0.25	3.3±0.15

DISCUSSION

At the first stage of the study of the immunotoxic action "Apiprost" capsules the ability of the drug to affect formation of cellular reactions in the DTH test was studied. The DTH reaction is aimed at determining the ability of the TS under study to affect the production of mediators that cause tissue infiltration with cellular elements by sensitized T-effectors. The introduction of antigen into the animal's pad leads to development of a local edema. According to the data obtained "Apiprost" capsules in the conditionally therapeutic dose (100 mg/kg) did not affect development of the DTH reaction – RI of animals from the experimental group was at the level of the immunized control. However, the increase of the dose to 500 mg/kg resulted in a statistically significant increase of RI. It indicates the stimulation of the cellular immune response to the antigen introduction. The data obtained are consistent with the literature data [6, 7, 5] on the stimulating properties of PPHD and BP, which are part of "Apiprost" capsules with respect to the immune system.

Determination of the effect of "Apiprost" capsules on the humoral immunity of animals has shown that TS in the dose of 100 mg/kg does not affect the processes of antibody formation. The immunostimulating effect of "Apiprost" capsules when using them in the dose of 500 mg/kg can be associated with the components of the drug – PPHD and BP that reveal the immunotropic effect [9]. Taking into account that "Apiprost" capsules are supposed to be used in clinics in the dose, which is equivalent to 100 mg/kg, it can be argued about the absence of immunotoxic effects of the drug studied.

The complex research conducted has allowed to determine that "Apiprost" capsules do not reveal the cumulative action, do not affect the genetic structures of somatic mammalian cells with a single and multiple-dose introduction, as well as do not cause genetic changes in the germ cells of the parent species, which lead to death of the first generation offspring at the embryonic stages of development.

CONCLUSIONS

1. The studies conducted to assess the cumulative properties have shown that intragastric administration of "Apiprost" capsules does not cause the drug accumulation in the body of the experimental animals.
2. The analysis of chromosomal aberrations of bone marrow cells in mice and rats has shown that "Apiprost" capsules do not have the mutagenic effect in the conditionally therapeutic

dose of 100 mg/kg (2.5-month introduction) and in the dose of 1000 mg/kg in a single administration). They do not cause genetic changes in the germ cells of the parent species as evidenced by the absence of death of the first generation offspring at the embryonic stages of development.

3. "Apiprost" capsules in the dose of 100 mg/kg do not affect the course of the delayed-type hypersensitivity reaction, do not change the number of plasmocytes in the spleen and the titer of hemagglutinins in the blood serum of immunized mice. The increase in the dose to 500 mg / kg has the immunostimulatory effect on the cellular and humoral immunity of animals.

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