UDC 619:615.324.076.9:638.167:57.086.132:612.017.12:636.932.028 DOI 10.36016/JVMBBS-2024-10-2-2

STUDY THE IMMUNOSTIMULATORY PROPERTIES OF A SOLUTION FOR INJECTION COMPRISING NATURAL POWDERED HONEY IN LABORATORY ANIMALS

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Summary. The article presents the results of pharmacological studies of a new domestic drug 'Apimel' in the form of a solution for injection based on natural powdered honey as an active ingredient. The effect of the solution for injections with natural powdered honey on the severity of the immune response in rats and mice with normal immune status was studied, and the immunostimulating properties of the drug 'Apimel' in mice with immunodeficiency were studied. It was found that the most pronounced immunostimulatory properties of the drug were observed in doses of 50 and 250 mg of natural powdered honey/kg body weight. The study of the effect of the drug on the development of a slow-type hypersensitivity reaction in mice revealed the anti-inflammatory properties of the drug at doses of 25 and 150 mg of natural powdered honey per kilogram of body weight. In the course of studying the immunostimulating properties of the investigated drug in mice with immunodeficiency, it was found that the solution for injection at a dose of 50 mg of natural powdered honey/kg body weight restored the processes of antibody formation at the level of the comparison drug, and significantly exceeded it in terms of the expression of phagocytic activity of neutrophils

Keywords: immunodeficiency, phagocytosis, mice, rats

Introduction. The phagocytic activity of polymorphonuclear leukocytes (PMNs) and reticuloendothelial cells plays a significant role in the body's defense against infection. The capacity of these cells to phagocytize and digest a wide range of microbes that enter the body represents a fundamental protective mechanism of the body (Kuznetsova, Babadzhan and Frolov, 2012). I. I. Mechnikov asserted that the phagocytic reaction is the most significant aspect of natural immunity (Ataman, 2012). It is widely acknowledged that the division of immunity into cellular and humoral components is somewhat arbitrary, given that antibody formation is impossible without the participation of T cells, which are the primary regulators of the immune system. Consequently, the immune response is the result of the cooperative interaction of T and B cells, which to a certain extent reflects the potential capabilities of both immune systems (Kuznetsova, Babadzhan and Frolov, 2012). The antigen processed by the macrophage is recognized by the T helper cell, which involves the B cell in antibody production.

One of the primary methods for maintaining the normal functioning of the immune system and restoring immunity in immunodeficiency states is the use of immunostimulants (Dale, Foreman and Fan, 1994; Babov, Gromov and Nikipelova, 2001; Kasianenko et al., 2020).

These include natural and synthetic substances that can stimulate the body's immune system. Currently, a large number of immunostimulants are used, however, they vary in their effectiveness and many other properties that determine their harmlessness, ease of use, cost-effectiveness, etc. (Butenko et al., 2001).

The most suitable and effective for the body are natural, so-called endogenous immunostimulants, which are based on substances involved in the regulation of immune processes in the human and animal body (Dale, Foreman and Fan, 1994; Bauer et al., 1989). Our country's apiaries produce a natural treasure rich in a full range of vitamins and amino acids, offering a highly effective means for rapid health improvement. Furthermore, the use of bee products for prevention and treatment is not only affordable but also completely natural in composition (Tykhonov et al., 2014).

Honey has been used as a healing agent for thousands of years. Its importance cannot be overstated, as it is a source of nutrients, rich in vitamins B_1, B_2, B_6, E, K, and C, containing folic acid and provitamin A — carotene (Tykhonov et al., 2014).

The well-known cough syrup with plantain and ivy has been shown to have a strong antiviral effect, helping to cope with seasonal acute respiratory viral infections and bronchopulmonary diseases. Thanks to honey, the syrup has a pleasant taste and smell (Kovalenko, 2019).

Experimental studies show that bee honey-based products have a distinct ability to stimulate the phagocytic activity of neutrophils and macrophages, thus increasing nonspecific immunity (Butenko et al., 2001).

It is important to note that special attention should be paid to the substance of natural powdered honey (NPH), the technology of which was developed at the D. P. Salo Department of Pharmacy Technology of Drugs of the National University of Pharmacy (Kharkiv, Ukraine) under the guidance of Prof. O. I. Tikhonov, Academician of the Ukrainian Academy of Sciences (Tykhonov et al.,

2014; Tikhonov, 2010). The substance was used as an active pharmaceutical ingredient to develop a new domestic drug in the form of a solution for injection, which was given the conventional name 'Apimel (Tykhonov et al., 2014).

In light of the above information, it would be beneficial to study the pharmacological properties of the developed drug 'Apimel'.

The study aimed to investigate the effect of a solution for injection with natural powdered honey on the severity of the immune response in rats and mice with normal immune status, as well as to study the immunostimulatory properties of 'Apimel' in mice with immunodeficiency.

Material and methods. Experimental studies were conducted at the Central Research Laboratory of the National University of Pharmacy (Kharkiv, Ukraine).

Experiments on animals were conducted following the recommendations of the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 2010/63/EU (CEC, 2010), and in accordance with Art. 26 of the Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' (VRU, 2006) and basic bioethical principles (Simmonds, 2017). The research program was reviewed and approved by the Bioethics Committee of the National University of Pharmacy (Kharkiv, Ukraine) under the current procedure.

During the experiments, the animals were kept in a vivarium at a temperature of 18–24 °C, a humidity of 50–60%, in natural day-night light conditions, in plastic cages, and on a balanced diet under current standards (Kozhemiakin et al., 2002).

To achieve this goal, the effect of the drug on the phagocytic activity of peripheral blood PMNs in rats and the development of a slow-type hypersensitivity reaction in mice was first determined (Freireich et al., 1966).

The ability to influence the phagocytic activity of PMNs was tested by the reaction with yeast.

The investigated solution for injection 'Apimel' (NPH concentration 2.5 wt. %) was administered to rats intramuscularly at doses of 50, 100, 150, and 250 mg NPH/kg body weight for 3 days using five groups of animals:

Group 1 — negative control: rats injected with intramuscular saline;

Groups 2–5: animals injected with NPH solution at doses of 50, 100, 150, and 250 mg NPH/kg body weight, respectively.

To assess the severity of the phagocytic activity of PMNs, the following indicators were calculated: the percentage of polymorphonuclear leukocytes that phagocytosed (Fi — phagocytic index) and the average number of yeast particles absorbed by one PMN (Fu — phagocytic number).

The state of cellular immunity against the background of the experimental drug administration was determined by the delayed-type hypersensitivity reaction (DTH) by the method of Kitamura (1980). This reaction is aimed at determining the ability of the test object to influence the production of mediators by sensitized T effectors that cause tissue infiltration by cellular elements. The injection of antigen into the paw pad of an animal leads to the development of local edema (Kuznetsova, Babadzhan and Frolov, 2012; Kitamura, 1980).

The following groups of animals were used in the experiment:

Group 1 — unimmunized (intact) control;

Group 2 — immunized control, animals that were injected with saline before and during the entire period of immunization with ram erythrocytes (RE);

Groups 3–6 — animals that were injected with 'Apimel' solution at doses of 25, 50, 100, and 150 mg NPH/kg body weight, respectively, before and during the entire period of immunization with RE.

The total period of administration was 6 days.

The animals were immunized via a single intraperitoneal injection of a suspension of freshly washed RE at a dose of 2×10^5 cells in a volume of 0.5 ml of sodium chloride saline per 20 g of body weight. On 5th day, the final dose of antigen (10^8 RE) was administered via injection under the aponeurotic plate of one of the hind limbs (experimental paw) in a volume of 0.02 ml per animal. The same volume of saline was injected into the contralateral paw (control paw). After 24 h, the animals were euthanized by an overdose of ether anesthesia, the feet of the hind limbs were amputated at the level of the tarsometatarsal joint, and they were weighed on a torsion balance.

The severity of the local reaction was assessed by calculating the ratio of the weight of the feet of the experimental and control paws in each group of animals. The reaction index (RI) was calculated using the following formula (1):

$$RI = \frac{M_{exp.paw} - M_{c.paw}}{M_{c.paw}} \times 100\% \quad (1)$$

were: $M_{exp.paw}$ — weight of the experimental paw, g;
$M_{c.paw}$ — weight of the control paw, g.

The experimental data were processed using variation statistics with the standard statistical software package Statistica v. 6.0. To obtain statistical conclusions, the Mann–Whitney test (for data that do not follow the normal distribution law) and parametric methods (Newman–Keuls method) were used. When comparing statistical samples, the significance level of $p < 0.05$ was accepted (Lapach, Chubenko and Babich, 2001).

The next stage of the study was to investigate the immunostimulatory properties of 'Apimel' in mice with immunodeficiency modeled by intraperitoneal single injection of hydrocortisone acetate (Hydrocortison-

Richter, Budapest, Hungary) at a dose of 250 mg/kg body weight (Shvets and Portugalov, 1979).

As a comparison drug, we used a pharmacologically analogous immunostimulant of natural origin 'Thymaline', a lyophilized solution for injection, manufactured by PJSC 'Biofarma' (Kyiv, Ukraine).

The immunostimulatory properties of the test drug were evaluated at doses of 50, 100, and 150 mg NPH/kg body weight. 'Thymaline' was administered at a dose of 100 mg/kg body weight, calculated using the coefficient of conversion of doses by body area from the average daily dose for humans of 10 mg/kg body weight, following the methodology described by Ulanova, Sidorov and Khalepo (1968). The study drugs were administered five days prior to the induction of immunodeficiency and throughout the duration of the experiment, spanning a total of 11 days. One day following the administration of hydrocortisone acetate, mice were immunized with ram erythrocytes to assess the status of the immune system. The antigen was injected intraperitoneally at a dose of 0.2 ml/20 g of body weight.

The following groups of animals were used in the study:

Group 1 — negative control: animals administered drinking water;

Group 2 — pathology control: animals injected with hydrocortisone acetate at a dose of 250 mg/kg body weight;

Groups 3–5 — animals treated with hydrocortisone acetate and injected with natural powdered honey in doses of 50, 100, and 150 mg NPH/kg body weight, respectively;

Group 6 — animals treated with hydrocortisone acetate were administered the comparison drug 'Thymaline' at a dose of 100 mg/kg body weight.

To assess the degree of immunodeficiency and the efficacy of the drugs on 5th day after immunization, animals were euthanized under light anesthesia (chloroform). In the blood serum of experimental animals, the indicators of the humoral immune response titers of hemolysins and hemagglutinins were determined by the method of serial dilutions in polystyrene plates. The agglutination reaction is based on the ability of antibodies (agglutinins) contained in the blood serum of immunized animals to adhere to ram erythrocytes in an isotonic solution of sodium chloride. To evaluate the nonspecific resistance of the mice, we examined the phagocytic activity of neutrophils in the peripheral blood. We calculated the following indicators: the percentage of neutrophils that phagocytosed (Fi — phagocytic index) and the average number of yeast fungi absorbed by one neutrophil (Fu — phagocytic number). To gain further insight into the physiological state of the mice, the weight of the body and organs of immunogenesis (thymus and spleen) was determined as an integral indicator.

The obtained experimental data were statistically processed by the method of variation statistics (arithmetic mean and its standard error ($M \pm m$) or median and upper and lower quartiles (Me (UpQ, LQ;) (M (min; max)). When applying the method of mathematical statistics, the significance level was set at $p < 0.05$. To draw statistical conclusions when comparing statistical samples of relative variables, the Newman–Keuls or Mann–Whitney tests were used (Lapach, Chubenko and Babich, 2001).

Results and discussions. *Investigation of the effect of a solution for injection with natural powdered honey on the phagocytic activity of PMNs.* The study demonstrated the capacity of 'Apimel' to stimulate phagocytosis. The solution exhibited the greatest activity at doses of 50 and 250 mg NPH/kg body weight. The results demonstrated that the drug, administered at a dose of 50 mg NPH/kg body weight, significantly increased the number of absorbed yeast cells compared to the negative control. Furthermore, the phagocytic index, defined as the number of phagocytosed PMNs, increased significantly at a dose of 250 mg NPH/kg body weight (Table 1).

Table 1 — Effect of NPH injection solution on phagocytic activity of rat peripheral blood neutrophils (Me (UpQ; LQ))

Animal groups	Indicators of phagocytosis	
	Fi	Fu
Negative control	41 (29; 46)	3.1 (2.7; 3.4)
'Apimel', 50 mg NPH/kg body weight	45 (43; 55)	3.7 (3.6; 3.8)*
'Apimel', 100 mg NPH/kg body weight	56 (56; 61)*	3.05 (3; 3.1)
'Apimel', 150 mg NPH/kg body weight	50 (39; 54)	3 (2.8; 3.0)
'Apimel', 250 mg NPH/kg body weight	65 (59; 69)*	3.5 (3.2; 3.6)

Note. * — differences are significant relative to the negative control, $p < 0.05$.

It should be noted that in terms of Fu, the activity of the test drug at a dose of 50 mg NPH/kg body weight significantly exceeds that of the drug at doses of 100–250 mg NPH/kg body weight, but in terms of the ability to stimulate PMNs to phagocytosis (phagocytic index), it is inferior to the dose of 250 mg NPH/kg body weight.

Investigation of the effect of a solution for injection with natural powdered honey on the development of a delayed-type hypersensitivity reaction in mice. Administration of 'Apimel' at doses of 50 and 100 mg NPH/kg body weight induced a normal immune response in mice (Table 2).

Table 2 — Effect of NPH solution for injection on the development of delayed-type hypersensitivity reaction in mice (Me (UpQ; LQ))

Animal groups	Dose, mg NPH/kg body weight	Number of animals in a group	RI
Intact (non-immunized) control	—	8	1.7 (0; 3.1)
Immunized control (RE)	—	8	7.2 (3.8; 10.4)*
'Apimel'	25	8	3.7 (1.1; 4.9)**
	50	7	7.4 (4.9; 8.8)*
	100	8	7.5 (3.1; 8.7)*
	150	8	3.5 (0.8; 5.8)**

Notes: * — differences are significant relative to the intact control, $p < 0.05$; ** — differences are significant compared to the immunized control, $p < 0.05$.

The study established the ability of the drug to suppress the development of an immune inflammatory response at doses of 25 and 150 mg NPH/kg body weight, as indicated by significantly lower values of the corresponding DTH reaction index.

Investigation of immunostimulatory properties of the solution for injections with natural powdered honey in mice with immunodeficiency. The data obtained in previous studies on the immunostimulatory effect of 'Apimel' solution for injection on animals with normal immune status served as a basis for determining the effectiveness of the drug in conditions of immunodeficiency in mice modeled by hydrocortisone acetate (Tables 3–5).

Table 3 — Effect of NPH solution for injection on phagocytic activity of peripheral blood neutrophils in mice with immunodeficiency modeled by hydrocortisone acetate (Me (UpQ; LQ))

Animal groups	n	Indicators	
		Fi	Fu
Negative control	11	25 (21; 28)	3.4 (2.4; 3.6)
Control pathology	10	17 (15; 18)*	3.3 (3.0; 3.8)
'Apimel', 50 mg NPH/kg body weight	11	28 (26; 33) **/***	3.5 (3.3; 4.3)
'Apimel', 100 mg NPH/kg body weight	10	15 (10; 26)#	3.3 (3.1; 3.9)
'Apimel', 150 mg NPH/kg body weight	10	17 (15; 21)*#	3.2 (2.1; 4.8)
'Thymalin', 100 mg/kg body weight	8	21 (17; 23)	3.8 (3.2; 5.1)

Notes: * — differences are significant relative to the values of the negative control, $p < 0.05$; ** — differences are significant relative to the values of control pathology, $p < 0.05$; *** — differences are significant in relation to the values of the comparison drug 'Thymaline', $p < 0.05$; # — differences are significant in relation to the values of the experimental drug at a dose of 50 mg NPH/kg body weight, $p < 0.05$.

Table 4 — Effect of NPH solution for injection on the indices of humoral immunity in mice with immunodeficiency modeled by hydrocortisone acetate (Me (UpQ; LQ)

Animal groups	n	Indicators	
		Hemolysins, log_2	Hemagglutinins, log_2
Negative control	11	7 (5; 9)	8 (7; 8)
Control pathology	10	1.5 (0; 3)*	1 (0; 3)*
'Apimel', 50 mg NPH/kg body weight	11	5 (3; 5)**	4 (3; 6)**
'Apimel', 100 mg NPH/kg body weight	10	4 (3;5)**	4 (3; 4)**
'Apimel', 150 mg NPH/kg body weight	10	4 (2;5)*/**	3 (3; 4)*/**
'Thymalin', 100 mg/kg body weight	8	5 (4; 5.5)**	3.5 (3; 4.5) */**

Notes: * — differences are significant relative to the values of the negative control, $p < 0.05$; ** — differences are significant relative to the values of control pathology.

Table 5 — Effect of NPH solution for injection on the dynamics of mass coefficients of mice organs under conditions of immunodeficiency modeled by hydrocortisone acetate ($M \pm m$)

Animal groups	n	Weight, g	Mass coefficients of the organs	
			Thymus	Spleen
Negative control	11	22.3 ± 0.55	0.32 ± 0.03	0.91 ± 0.05
Control pathology	10	17.8 ± 0.57*	0.13 ± 0.013*	0.48 ± 0.17*
'Apimel', 50 mg NPH/kg body weight	11	17.7 ± 0.54*	0.23 ± 0.02*/**	0.29 ± 0.03*
'Apimel', 100 mg NPH/kg body weight	10	18.6 ± 0.54*	0.16 ± 0.012*	0.31 ± 0.03*
'Apimel', 150 mg NPH/kg body weight	10	18.7 ± 0.45*	0.18 ± 0.02*	0.38 ± 0.06*
'Thymalin', 100 mg/kg body weight	8	17.6 ± 0.34*	0.20 ± 0.02*	0.27 ± 0.03*

Notes: * — differences are significant relative to the values of the negative control, $p < 0.05$; ** — differences are significant relative to the values of control pathology.

The administration of hydrocortisone acetate to mice of the control pathology group led to the development of immunodeficiency, which was reflected in impaired

phagocytosis and decreased antibody production and was accompanied by a decrease in both body weight and mass coefficients of the thymus and spleen of mice, which is a natural result of the cytotoxic effect of high doses of hydrocortisone acetate.

The results of the study on the effect of the solution for injection with natural powdered honey on the phagocytic activity of polymorphonuclear leukocytes of peripheral blood of rats and the development of a delayed-type hypersensitivity reaction in mice indicate that the most pronounced stimulating properties of the drug 'Apimel' are found in doses of 50 and 250 mg NPH/kg body weight. Upon analysis of the data from the study on the effect of the solution for injection with natural powdered honey on the development of a delayed-type hypersensitivity reaction in mice, it was determined that 'Apimel' in doses of 25 and 150 mg NPH/kg body weight suppresses the immune inflammatory reaction provoked by the introduction of RE, indicating the anti-inflammatory properties of the drug. At doses of 50 and 100 mg NPH/kg body weight, the solution for injection did not affect the normal immune response of mice to antigen administration.

A study of the immunostimulatory properties of the solution for injections with NPH in mice with immunodeficiency revealed a notable decline in the phagocytic activity of peripheral blood neutrophils. The number of phagocytic neutrophils was found to be 1.5 times lower than that of the negative control (Drogovoz et al., 2010). However, the phagocytic number remained at the level of the negative control (Table 3). At the same time, the process of antibody formation was impaired, as evidenced by a significant decrease in the titers of hemolysins and hemagglutinins in the serum of animals by 78 and 88%, respectively (Table 4).

The administration of hydrocortisone acetate to mice resulted in the development of secondary immunodeficiency, characterized by impaired nonspecific resistance and antibody formation in animals. Conversely, prophylactic intramuscular injection of the test drug 'Apimel' led to the restoration of animal immunoreactivity, with hemolysins' and hemagglutinins' titers exceeding those of the control group by an average of 3–4 times. The solution for injection with NPH demonstrated the most pronounced effect at a dose of 50 mg NPH/kg body weight. In animals injected with the drug at a dose of 50 mg NPH/kg body weight, both antibody production and the phagocytic activity of neutrophils were increased to the level of the negative control (Tables 3, 4). Furthermore, at a dose of 50 mg NPH/kg body weight, the drug demonstrated a unique effect of increasing thymic mass coefficient, in contrast to the other experimental groups (Table 5). Meanwhile, spleen mass coefficient remained significantly lower than the values observed in the negative control group (Jerne and Nordin, 1963).

It is important to highlight that the 'Apimel' solution for injection, at a dose of 50 mg NPH/kg body weight, demonstrated comparable efficacy to the reference drug in restoring antibody formation processes. However, it significantly exceeded the reference drug in terms of the phagocytic activity of neutrophils (Table 3). Our studies have proven the potential of 'Apimel' solution for injection as a viable treatment option.

Conclusions. The solution for injection with natural powdered honey, when administered intramuscularly to rats in the dose range of 50–250 mg NPH/kg body weight, has been observed to demonstrate moderate phagocytic activity.

'Apimel' has been shown to stimulate the phagocytic activity of PMNs most effectively at doses of 50 and 250 mg NPH/kg body weight. At doses of 25 and 150 mg NPH/kg body weight, the test drug demonstrated efficacy in suppressing the development of the DTH reaction in mice, indicating the anti-inflammatory properties of the drug.

The solution for injection with natural powdered honey exhibited notable immunostimulatory properties in conditions of immunodeficiency induced by hydrocortisone acetate. Its efficacy was comparable to that of 'Thymalin', a well-known immunomodulator of natural origin.

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