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# DEVELOPMENT OF A MOUSE MODEL OF ALLERGIC RHINITIS AND BRONCHIAL ASTHMA CAUSED BY WORMWOOD POLLEN

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**Abstract**

**Relevance.** The prevalence of IgE-mediated allergic diseases among the population of industrially developed countries has reached 35%, and experts estimate that it will increase further in the next decade. Allergen-specific immunotherapy (SIT) is the only therapeutic approach capable of changing the natural course of the allergic process. Global prevalence of allergic diseases and several disadvantages of commercially available SIT products, made us start the search for new and improved specific treatment of allergies, which in turn requires the proper modeling of allergic diseases using laboratory animals.

**Aim.** Modeling of IgE-mediated bronchial asthma and allergic rhinitis in mice for future development of a new vaccine for allergen-specific immunotherapy.

**Materials and Methods.** Study design – experimental preclinical prospective cohort study. The entire cycle of work with laboratory animals was carried out at M. Aikimbayev National Research Center for Especially Dangerous Infections of the Ministry of Health of the Republic of Kazakhstan from September 2020 to January 2021. To model IgE-mediated bronchial asthma 8-12 week old SPF Balb/c female mice were used. Mice were sensitized by intranasal (IN) and intraperitoneal (IP) ways with 3 different extracts: bitter wormwood, common wormwood and their mix. Our choice of these particular allergens is determined by their significance in the ethiology of seasonal pollinosis in Kazakhstan. When assessing the level of sensitization of mice, a comprehensive approach was used, which included the analysis of antibody and cytokine levels, cellular immune responses, clinical signs and histological analysis of the lungs. Generally accepted methods of statistical processing of experimentally obtained samples of varying variables were used. The mean value of the sample (X) and the standard error (m) were determined. The reliability of the differences between the indicators and the groups was determined using the Graph Pad Prism 8 statistical program (Graph Pad Software, Inc., La Jolla, CA, USA).

**Novelty:** Modeling of allergic rhinitis and bronchial asthma with IN and IP regimes of sensitization with the use of bitter wormwood and common wormwood extracts and their mix in mice was tested for the first time.

**Results:** Among the allergens tested, common wormwood extract in the intraperitoneal sensitization had shown the most potent sensitizing effect: high values of total and antigen-specific IgE antibodies; pronounced polarization of immune response toward Th2 by the ratio of IgG1/IgG2a antibodies; positive reaction in the allergy ear swelling test; induced perivascular and eosinophilic inflammation in the lungs with expression of the allergic proinflammatory cytokine IL-5.

**Conclusions:** In modeling of IgE-mediated bronchial asthma in mice, common wormwood extract with intraperitoneal sensitization has the most potent sensitizing effect.

***Keywords:*** *allergy, bronchial asthma, sensitization, mouse, wormwood.*

**Резюме**

# РАЗРАБОТКА МЫШИНОЙ МОДЕЛИ АЛЛЕРГИЧЕСКОГО РИНИТА И БРОНХИАЛЬНОЙ АСТМЫ, ВЫЗЫВАЕМЫЕ ПЫЛЬЦОЙ ПОЛЫНИ

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**Актуальность.** Распространенность IgE-опосредованных аллергических заболеваний среди населения промышленно развитых стран достигло 35%, и, по оценкам экспертов прогнозируется их дальнейшее увеличение уже в следующем десятилетии. Единственным терапевтическим подходом способным изменить естественное течение аллергического процесса является АСИТ. Глобальная превалентность аллергических заболеваний и не совершенность коммерчески доступных АСИТ, обуславливает необходимость поиска новых или усовершенствование существующих способов специфической терапии аллергии, который в свою очередь, требует правильное моделирование аллергических заболеваний на лабораторных животных.

**Цель.** Моделирование IgE-опосредованной бронхиальной астмы и аллергического ринита у мышей для будущей разработки новой вакцины для аллерген-специфической иммунотерапии.

**Материалы и методы.** Дизайн исследования: экспериментальное-доклиническое проспективное когортное исследование. Весь цикл работ с лабораторными животными выполнялся в Национальном научном центре особо опасных инфекции им. М. Айкимбаева МЗ РК с сентября 2020 по январь 2021 года. Для моделирования IgE-опосредованной бронхиальной астмы были использованы 8-12 недельные SPF Balb/c мыши - самки, которые были сенситизированы интраназальным (ИН) и интраперитонеальным (ИП) путем экстрактами полыни горькой и обыкновенной, а также их миксом. Выбор данных аллергенов обуславливается их значимостью в наибольших случаях сезонного поллиноза в Казахстане. При оценке уровня аллергизации мышей был использован комплексный подход, который включал изучение антительного, цитокинового, клеточного иммунного ответов, клинических признаков и гистологический анализ легких. Использовались общепринятые способы статистической обработки экспериментально полученных выборок варьирующих переменных. Определяли среднее значение выборки (X), среднеквадратичную ошибку (m). Достоверность различий между показателями и группами определяли с использованием статистической программы Graph Pad Prism 8 (Graph Pad Software, Inc., La Jolla, CA, USA).

**Новизна.** Впервые разработана мышиная модель аллергического ринита и бронхиальной астмы с ИН и ИП режимами сентитизации и использованием экстрактов полыни горькой и обыкновенной, а также их микса.

**Результаты.** Среди испытанных аллергенов наиболее аллергизирующим действием обладал экстракт полыни обыкновенной, который при ИП режиме сентитизации формировал высокие значения общих и антиген-специфичных IgE антител, индуцировал выраженную поляризация иммунного ответа в сторону Th2 по соотношению IgG1/IgG2a антител, вызывал положительную реакцию в ушном тесте, периваскулярное и эозинофильное воспаление в легких с экспрессией аллергического провоспалительного цитокина IL-5 в легких животных.

**Выводы.** При моделировании IgE-опосредованной бронхиальной астмы и аллергического ринита у мышей наиболее аллергизирующим действием обладает экстракт полыни обыкновенной при ИП режиме сенситизации.

***Ключевые слова:*** *аллергия, бронхиальная астма, сенситизация, мышь, полынь.*

**Түйіндеме**

# ЖУСАН ТОЗАҢЫМЕН ШАҚЫРЫЛАТЫН АЛЛЕРГИЯЛЫҚ РИНИТ ПЕН БРОНХИАЛДЫ ДЕМІКПЕНІНІҢ ТЫШҚАН МОДЕЛІН ЖАСАУ

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**Өзектілігі.** Индустриалды дамыған елдердің тұрғындары арасында аллергиялық аурулардың таралуы 35%-ға жетті және сарапшылардың бағалауы бойынша келесі онжылдықта олардың одан әрі өсуі болжануда. АСИТ-аллергиялық процестің табиғи ағымын өзгерте алатын жалғыз терапиялық тәсіл. Аллергиялық аурулардың жаһандық таралуы және коммерциялық қол жетімді АСИТ препараттарының жетіксіздігі аллергияны емдеудің жаңа әдістерін іздеуді немесе қолданыстағы әдістерді жетілдіруді қажет етеді, бұл өз кезегінде зертханалық жануарларда аллергиялық ауруларды дұрыс модельдеуді қажет етеді.

**Мақсаты:**. Аллерген спецификалық иммунотерапияға арналған жаңа вакцинаны болашақта әзірлеу үшін тышқандардағы аллергиялық бронхиалды демікпені және аллергиялық ринитті модельдеу.

**Материалдар мен әдістер:** Зерттеу дизайны: эксперименттік-клиникаға дейінгі перспективалық когорттық зерттеу. Зертханалық жануарлармен жұмыстың барлық циклі 2020 жылғы қыркүйектен 2021 жылғы қаңтарға дейін ҚР ДСМ М. Айқымбаев атындағы Аса қауіпті инфекциялар ұлттық ғылыми орталығында (АҚИҰҒО) орындалды. IgE-ассосацияланған бронхиалды демікпені модельдеу үшін 8-12 апталық SPF BALB/c ұрғашы тышқандары пайдаланылды. Тышқандар ащы және қарапайым жусан сығындылары, сондай-ақ екеуінің араласқан түрін пайдалана отырып, интраназальды (ИН) және интраперитонеальді (ИП) тәсілдермен сенситизацияланды. Бұл

аллергендерді таңдау олардың Қазақстандағы маусымдық поллиноздың ең үлкен жағдайларында себепкер болуына байланысты. Тышқандардағы аллергия деңгейін бағалау кезінде антиденелерді, цитокиндерді, жасушалық иммундық жауаптарды, клиникалық белгілерді және өкпенің гистологиялық талдауын зерттеуді қамтитын кешенді тәсілдер қолданылды. Өзгермелі айнымалылардың эксперименттік алынған үлгілерін статистикалық өңдеудің жалпы қабылданған әдістері қолданылды. Үлгінің орташа мәні (X), орташа квадраттық қате (m) анықталды. Көрсеткіштер мен топтар арасындағы айырмашылықтардың дұрыстығы Graph Pad prism 8 (Graph Pad Software, Inc., La Jolla, CA, USA) статистикалық бағдарламасын қолдану арқылы анықталды.

**Жаңалығы:** Алғаш рет ащы және қарапайым жусан сығындыларымен, сондай-ақ екеуінің араласқан түрін пайдалана отырып, интраназальды және интраперитонеальды әдістермен аллергиялық ринит пен аллергиялық бронх демікпені лабораториялық тышқанда модельденді.

**Нәтижелер:** Сыналған аллергендердің ішінде ең аллергизациялайтын әсерге интраперитонеальды сентитизация режиміндегі қарапайым жусан сығындысы ие болды: жалпы және антиген спецификалық IgE антиденелерінің жоғарылауы, IgG1/IgG2a антиденелер қатынасы бойынша иммундық жауаптың Th2-ге айқын поляризациясын тудырды, құлақ сынағында оң реакция тудырды, өкпеде IL-5 аллергиялық қабыну цитокинінің экспрессиясымен жүретін периваскулярлық және эозинофильді қабынуы байқалды.

**Қорытынды:** Тышқандарда аллергиялық бронх демікпесі мен аллергиялық ринитті модельдеу кезінде интраперитонеальды сентитизация режимінде қарапайым жусан сығындысы ең аллергизациялайтын әсерге ие болды.

***Түйінді сөздер:*** *аллергия, бронх демікпесі, сенситизация, тышқан, жусан.*

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**Introduction**

There is a high prevalence of immunoglobulin E (IgE)-mediated allergic diseases in industrialized countries involving over 1 in 3 people [23] and experts predict a further increase in the next decade [4,6,8]. Given the severity of the consequences of seemingly harmless allergic rhinitis (AR), the strategy of its prevention and management must be clearly defined. The most common complication of allergic rhinoconjunctivitis is bronchial asthma (BA), affecting 10-20% of children and adults [11,12].

Wormwood pollen (Artemisia) is one of the ten global aeroallergens, that most often cause bronchial asthma [19]. For this reason we used the two most common species of wormwood and their mix responsible for the largest cases of seasonal pollinosis in Kazakhstan. Considering that in many countries [17,21,23,24] including Kazakhstan [22], the wormwood flowering period (from July to October) is long enough, the quality of life of patients suffers for a long time.

The best strategy for treating allergic diseases is a combined approach: elimination of the allergen, patient education, adequate pharmacotherapy and allergen-specific immunotherapy (SIT) [11,12].

There is no doubt that the elimination method is the most effective. Even a small reduction in the concentration of allergens in daily life can significantly reduce the severity of clinical manifestations and reduce the amount of medications consumed. However, it is often impossible to completely avoid contact with an allergen [10].

Standard pharmacotherapy can achieve symptom control but provides only temporary relief and may also bring various side effects [1,10]. In addition, despite taking medication regurlarly, 70% of patients report an increase in the severity of clinical manifestations over time, an increased need for medication, and a decrease in quality of life [2,25]. In 18-48% of cases, symptom control is not achievable even with adequate pharmacotherapy [25].

The only therapeutic approach capable of changing the natural course of the allergic process is AIT [17]. SIT doesn't just reduce the severity of clinical manifestations and improve the quality of life, but it also demonstrates long-term efficacy that can persist for years after treatment completion [18]. AIT has a real therapeutic effect by restructuring unwanted allergen-specific humoral and T-cell immune responses from Th2 to mixed Th1/T Reg [5].

Global prevalence of allergic diseases and, at the same time, the imperfection of commercially available SIT, necessitates the search for new or improved methods of specific therapy of allergies. At the current stage of medical science, a considerable part of knowledge about the mechanisms underlying a particular disease and its treatment has been obtained using experimental models, which can be applied to various human diseases. In vivo studies are a fundamental step in the research of the safety and efficacy of new therapeutic agents.

A number of experimental models of asthma in animals have been described in the literature, which are used to study various aspects of pathogenesis and to approve new

methods of asthma treatment [5,9,16,17,19,20] But mice have become the most popular in modeling allergic processes in the respiratory tract. The predominant reason of using mice for modeling of asthma is certain similarities between the parameters of mouse models of allergic asthma and those of human asthma, including morphological changes in the airways and lung tissue, nature of the antigenic response and effector cells and molecules involved in the development and maintenance of allergic inflammation [3]. Currently, science has sufficient indisputable evidence of homology between human and mouse immune systems, which makes mice attractive for researches of the immune mechanisms of allergic asthma [7,17].

According to foreign literature, BALB/c, C57BL/6, A/J, C3H/HeJ, SWR, FVR, DBA/2 mouse lines are the most susceptible to sensitization and suitable for modeling allergic diseases [13]. Among the aforementioned lines, BALB/c and C57BL/6 are the most commonly used to model IgE-dependent asthma. BALB/c mice have found wide application for the study of induced bronchial hyperresponsiveness. They are characterized by high IgE- and IgGl-response to allergen administration, pronounced bronchial hyper responsiveness to intravenous and inhalation administration of methacholine, and significant amount of eosinophils in bronchial-alveolar lavage [9,14,15]. The choice of the right mouse line is an important factor in achieving the goals of the experiment.

In addition, it is important to remember that in order to obtain optimal immunological parameters (IgE/IgG1) and the response of the bronchial tract to the allergen, it is necessary to use young mice, the optimal age of 8-12 weeks. At this age, mice are more susceptible to the effects on the respiratory and immune systems [9,14].

Mice are and are likely to remain the most popular animals in asthma modeling, not least because of the economical and physiological advantages. Mice have very short reproductive cycle, which allows quicker start of the study; lower maintenance costs compared to other laboratory species and are physically more convenient to use in the study due to their low body weight and diminutiveness; and lastly, their overall aggressiveness is low. And one cannot ignore the fact that, historically, mice have become an effective mean of generating hypotheses for subsequent testing in humans. Obviously, for preclinical studies of the properties of allergy vaccines and their therapeutic efficacy modeling of IgE-dependent asthma in mice is nothing but adequate.

**Aim.** Modeling of IgE-mediated asthma and allergic rhinitis in mice for future development of a new vaccine for allergen-specific immunotherapy.

**Materials and methods.**

Study design- experimental preclinical prospective cohort study. The entire cycle of work with laboratory animals was carried out at the M. Aikimbayev National Research Center for Especially Dangerous Infections of the Ministry of Health of the Republic of Kazakhstan from September 2020 to January 2021. **(**Figure 1).

**Figure 1. Keeping SPF Balb/c mice in microisolators.**

Specific pathogen-free (SPF) inbred BALB/s mice from Envigo were used to model asthma (total number of mice – 70: 6 experimental groups of 10 mice and 2 control groups of 5 mice); health certificates are available. In 2019, animal health was monitored by the accredited ICLAS (International Council for Laboratory Animal Science) company in accordance with the requirements of FELASA (Federation of Laboratory Animal Science Associations).

*Intranasal sensitization of mice*

For this purpose, 8-12 weeks old SPF Balb/c mice (female; n=10/group, 30 mice in total) were injected under ether anesthesia with extracts of pollen of bitter wormwood, common wormwood and their mix (in the ratio 1: 1, Burly, Almaty, Kazakhstan) at a concentration of 200 PNU/20 µL (protein nitrogen unit) or PBS alone in control group (20 µL, n=5) for 6 consecutive days during the first and second weeks and 4 consecutive days during the third week (days 0-5, 7-12 and 14-17).

*Intraperitoneal sensitization of mice*

8-12 weeks old SPF Balb/c mice (female, n=10/group, 30 mice total) were intraperitoneally injected twice at 14 day intervals with an extract of bitter pollen, common pollen and their mix (in the ratio 1: 1) in concentrations of 1000 PNU/200 µL (Burly, Almaty, Kazakhstan) sorbed on aluminum hydroxide (Alhydrogel® adjuvant 2%, InvivoGen, USA; final concentration of aluminum ions in suspension was 5 mg/ml) or PBS alone in control group (200 µL, n=5).

*Intranasal challenge of mice*

Mice were challenged three times on days 37, 39, 41 after the beginning of sensitization by intranasal inoculation of allergen extracts (200 PNU/20 µl) in experimental groups or PBS alone in control group (20 µL).

*Clinical observation of mice*

After the third challenge with allergen extracts, the signs of allergic manifestations (nose rubbing, sneezing, scratching the ear where the allergen was injected, tail biting) were evaluated in mice for 10 min, which were quantified for each individual sign and in their aggregate.

*Allergy Ear Swelling Test*

After a course of sensitization and challenge, mice in experimental groups were intradermally injected with 10 µl (100 PNU) allergen extracts only into the right auricle and negative control group received the PBS. After 1.5-2 hours, the thickness of both auricles was measured using an electronic digital micrometer MCC-25 DSWQ0-100II (China). Results are presented as the difference in thickness of the right (allergen injection) and left (no injection) auricles expressed in mm.

*Blood sampling*

On days 24 (before challenge) and 48 (after challenge) blood samples were collected from mice for testing for total IgE antigen-specific (to major recombinant protein Art v 1) IgE, and IgG1/IgG2a and IgA antibodies by ELISA.

*Euthanasia and organ harvesting.*

The mice were euthanized by cervical dislocation and organs were harvested for analysis: the level of lung inflammation in mice (at day 41) was determined by histological analysis, as well as by determining pro-inflammatory cytokines (IL-5 and IL-6) in lung homogenates (IgA antibodies were additionally determined). To evaluate allergen-induced immune response shifts, spleen was harvested to determine CD4+/CD8+ cells and cytokine profile (IL-4, IFN-gamma, IL-2, IL-10, IL-5, IL-6).

Immunological and histological methods of investigation were used:

*ELISA.*

96-well microplates were immobilized with 5 µg/10 mL (plate) of recombinant Art v 1 protein on commercial buffer (ELISA Coating Buffer, BioLegend) overnight. The next day, blocking solution (ELISA Assay Diluent, #421203, BioLegend) was poured into the plates in PBS at 200 µl/well and incubated under constant shaking (300-330 rpm on a PST-60HL thermal shaker, BIOSAN) for 1 h at room temperature. The plates were then washed four times with ELISA Wash Buffer (# 421601, BioLegend) in PBS. Mouse serum samples were diluted 1:5 (for IgE, IgG2a, IgA antibodies) or 1:1000 (IgG1 antibodies) with blocking solution (for IgA antibody determination, lung homogenate supernatant was introduced whole) 100 µl into wells and incubated under constant shaking (300-330 rpm) for 1.5-2 h at room temperature with stirring. After washing (4x), anti-mouse biotinylated detection antibodies for IgE, IgG1, IgG2a, IgA (1:200, BioLegend, 100 µl/well) were added and the plates were incubated (1 h at room temperature with stirring). After an additional wash (4x), plates were incubated with HRP Streptavidin (#405210, BioLegend, 1:1000, 100 µl/well) for 30 min at room temperature with stirring. The wash (5x) was then repeated, at the end of which the TMB substrate (100 µl/well) was poured. The color reaction was stopped by adding 2.5 M H2SO4 (100 µl/well), and the optical density was measured (measuring wavelength 450 nm, reference wavelength 630 nm) on a Stat Fax 2100 analyzer (Awareness Tech).

*Assessment of Cytokine Profile*

Mice were euthanized (cervical dislocation under ketamine/xylazine anesthesia) Harvested spleens were mechanically crushed into a single cell suspension using a cell strainer (Falcon® 70µm Cell Strainer). The shredding procedure was performed on a Petri dish with 10 ml of 3% FBS (fetal bovine serum)-FBR. Erythrocytes were lysed with lysis buffer (150 mM NH4Cl, 1 mM CHCO3, 0.1mMNa2 EDTA, pH 7.3). Splenocytes were cultured in a 5% CO2 incubator at 37°C in 24-well flat-bottomed plates at 1×106 cells/well (1 ml) in RPMI-1640 medium with 20 mM HEPES and L-glutamine (R7388-1L, Sigma) and 10% fetal bovine serum (#TMS-013-B, Sigma) inactivated by heating in the presence of 2 µg of purified recombinant Art v1 protein (Mugwort Major pollen allergen Art v 1 protein, N-His Tag, AtaGenix) or without protein (control without stimulation). Cells were incubated for 72 hours, after which the supernatant was examined for cytokines IL-4, IFN-gamma, IL-2, IL-10, IL-5, and IL-6 using commercial ELISA MAX™ Deluxe Set Mouse kits (BioLegend), according to the manufacturer's instructions. The remaining cells were used to identify CD4+ and CD8+ T-lymphocytes on a flow cytofluorimeter.

*Cytofluorimetric cell analysis*

Cell phenotype was assessed by determining CD markers using flow cytofluorimetry. For this purpose, cells were incubated with antibodies specific to surface markers for 20 min in the dark on ice. The following fluorochrome-labeled antibodies were used: PE anti-CD8a, FITC-anti-CD4 (Biolegend, USA) at a concentration of 0.25 µg/100 µl of cell suspension of splenocytes. After incubation, cells were resuspended in PBS. At least 50x105 cells were analyzed for each sample on an Attune NxT flow cytometer (Thermo

Fisher, USA) using Attune NxT Software (Thermo Fisher, USA). T-cell populations were analyzed in the lymphocyte gating isolated on an FCS/SSC dot-plot. The results were presented as the ratio of CD4+ to CD8+ cell counts.

*Histological analysis*

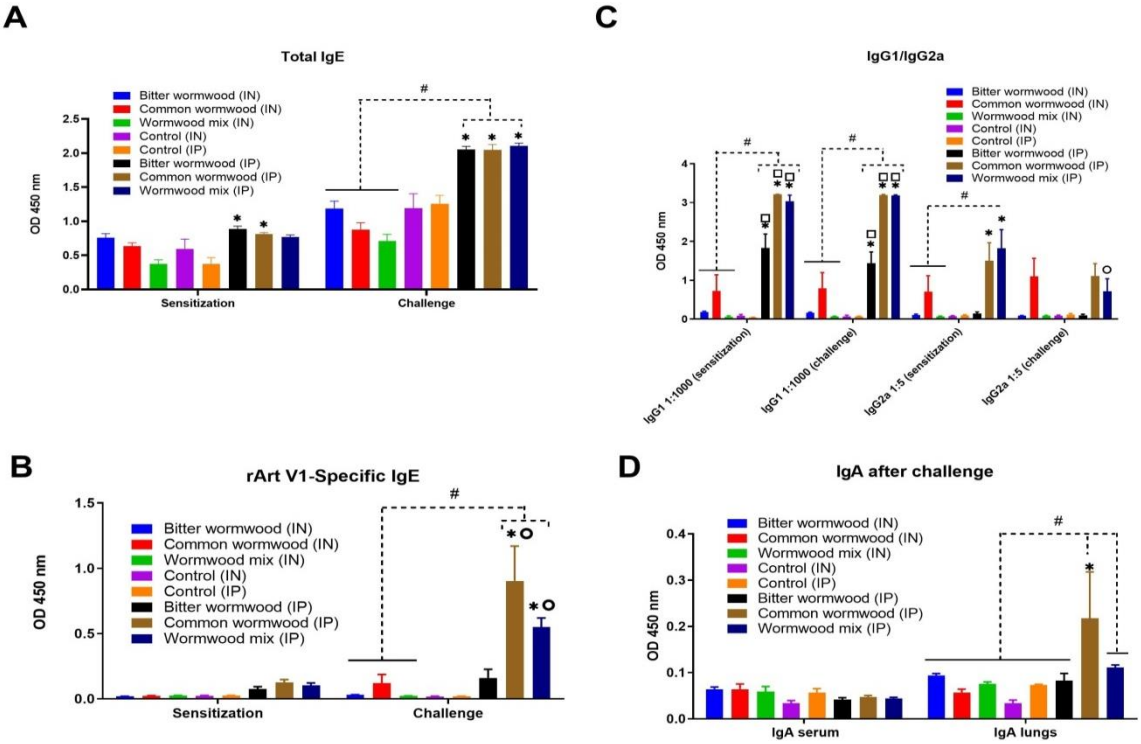
The degree of perivascular inflammation, as well as eosinophilic infiltration in the lungs, was determined by the score system described in [25]. For tissue histology, mice were euthanized by cervical dislocation. The lungs were restored and fixed in 10% formaldehyde and placed in paraffin blocks. The sections were stained with hematoxylin and eosin to determine cellular infiltrates. A semi-quantitative assessment of both perivascular inflammation and eosinophilic infiltration was carried out. A normal lung (without inflammation) was evaluated (-); low scores (+) were assigned to multifocal perivascular infiltrates. The average score (++) was given to sections with more diffuse infiltrates. The mark (+++) is given to sections with pronounced and diffuse inflammation of the perivascular vessels. The number of eosinophils was estimated for each mouse at a 40-fold increase in 10 fields randomly selected throughout the lung. The results are expressed as an average count per group, the number of crosses was expressed in the corresponding figures.

*Animal housing and bioethics.*

The studies in laboratory animals were performed in the M. Aikimbayev National Research Center for Especially Dangerous Infections of the Ministry of Health of the Republic of Kazakhstan. Microisolator technology in individually ventilated complexes (Labproduct & Allentown, USA) were used to raise SPF animals. Animals were provided with feed and water *ad libitum* with optimal environmental conditions: air temperature 20-24°C, humidity 45-65%, illumination 325-350 Lx, noise level - no more than 60 dB, air volume per animal 0.25 m3 /h, airflow rate 0.2 m/s, number of animals per cage no more than 10, minimum cage area 180 cm2, full-fed food for adult animals - 12 g/head/day, for young animals - 5-8 g/head/day. Laboratory animals were provided with daily veterinary supervision, ability to meet physiological and behavioral needs, and rapid elimination of factors that could cause stress and suffering to the animals. Autoclaved granulated feed, SSNIFF, standardized, enriched feed with vitamins, amino acids, and minerals (62 elements), with at least 19-22% crude protein, without animal and growth supplements, without antibiotics, with ISO 9001 quality certificate were used. Rehofix MK-2000 (JRS, Germany) autoclavable, dust-free, made of natural materials, with high absorption capacity, compatible with ventilated complexes, were used as bedding material. Studies with laboratory mice were conducted according to Protocol #3 dated June 16, 2020, approved by the Institutional Committee on the maintenance and use of laboratory animals of the M. Aikimbayev National Research Center for Especially Dangerous Infections.

*Statistical analysis.* Common methods of statistical processing of experimentally obtained samples of varying variables were used. The mean value of the sample (X) and the standard error of the sample (m) were determined. Significance of differences between the variables and groups was determined using Graph Pad Prism 8 statistical program (Graph Pad Software, Inc., La Jolla, CA, USA). A P value < 0.05 was considered significant.

**Results.** *ELISA.* The results of the studies showed (Fig. 2A and B) that IN and IP regimes of sensitization in mice lead to the formation of both general and antigen-specific (to the major recombinant protein Art V1) allergic IgE antibodies.




IN - intranasal; IP - intraperitoneal, (*) P = 0.03-< 0.0001 to the corresponding control group;
(□) P = 0.0036-<0.0001 to the corresponding class of IgG2a antibodies;
(o) P = 0.0096-<0.0001 to the corresponding sensitization group; # P = 0.0272-<0.0001

**Figure 2. Specific antigenic response in SPF BALB/c mice after sensitization and allergic challenge with wormwood extracts.**

At the same time, the most significant values of total IgE antibodies were observed in the groups of mice with the IP sensitization regime (bitter and common wormwood extracts, P=0.018-0.0026 versus the corresponding control groups). IN challenge of mice with allergen extracts resulted in a more than significant increase in total and antigen-specific IgE antibody levels in the groups of animals with IP sensitization regime (P=0.0015-<0.0001 vs. corresponding control groups), which were significantly higher than in the groups with IP sensitization regime (P=0.0015-<0.0001 corresponding group with IP sensitization). It is important to emphasize that antigen-specific IgE antibodies, even after challenge, were not significant (P=0.95) in the group of mice with IP sensitization, where a bitter wormwood extract was used, compared with the corresponding control group. We first attributed this fact to the major recombinant protein Art V1, which probably has less cross-reactivity with bitter wormwood extract. However, even when using the bitter wormwood extract as an antigen in the ELISA, we had identical results.

Antigen-specific IgG1 (P=0.028-<0.0001) and IgG2a (P=0.026-<0.0001) antibodies were also more higher with IP sensitization regime compared with IN sensitization (Figure 2C).

The levels of IgG1 (P<0.0001) and IgG2a (P=0.014-0.0012) antibodies in the IP sensitization regime not only had significant values compared with the respective control groups, but also had a pronounced polarization toward the Th-1 immune response (dominance of IgG1 isotype antibodies over IgG2a, P=0.0036-<0.0001). It should be noted that among the groups of mice with IP sensitization, the values of IgG1 and IgG2a antibodies were different, and were least formed in animals injected with bitter wormwood extract (P=0.016-<0.0001 with respect to other experimental groups after sensitization). IN challenge of mice with allergen extracts had no effect on the level of antigen-specific IgG1 antibodies. However, it was noteworthy that the level of IgG2a antibodies in the IP mice with the sensitization regime (common wormwood and wormwood mix) after challenge was not significant (P=0.83-> 0.999) relative to the corresponding control groups. This fact indicates that after challenge, the Th1 polarized immune response was enhanced in the above groups of animals.

At the same time, levels of antigen-specific IgA type antibodies in serum and lung homogenates (1/2 of a lung in 1 ml of RPMI) were determined in sensitized mice after challenge (Figure 2D). Significant accumulation of IgA antibodies was detected only in the lungs of mice sensitized with the IP regimen using common wormwood extract (P=0.006 versus corresponding control; P=0.02-<0.0001 versus other experimental groups).

*Cytokine profile and immunogram of mice after challenge*

After challenge with allergen extracts, all mice were euthanized and spleen samples were taken to evaluate the antigen-specific cytokine profile and to detect CD4+ and CD8+ T-lymphocytes. A total of 6 most significant cytokines (IL-4, IFN-gamma, IL-2, IL-10, IL-5, IL-6) were assessed in splenocytes in response to rArt V1 protein stimulation. We found (Figure 3) no significant (P>0.05) cytokine expression compared with the corresponding controls, i.e., no shift toward the Th2 immune response was observed.

On the contrary, when evaluating the CD4+/CD8+ cell ratio (Figure 4), a pronounced (P=0.041 versus the corresponding control) polarization toward Th2 immune response was observed in the group with IP sensitization regime using the wormwood mix, which was significantly higher (P=0.034-<0.0001) than in all other experimental groups.

*Clinical observation of mice after sensitization and challenge*

The manifestation of allergic reactions in mice after challenge was assessed by the characteristic clinical signs of rhinitis (nose rubbing, sneezing) and other additional manifestations (scratching the ear where the allergen was injected; tail biting). The presence of allergies was additionally confirmed using an intradermal allergy ear swelling test.

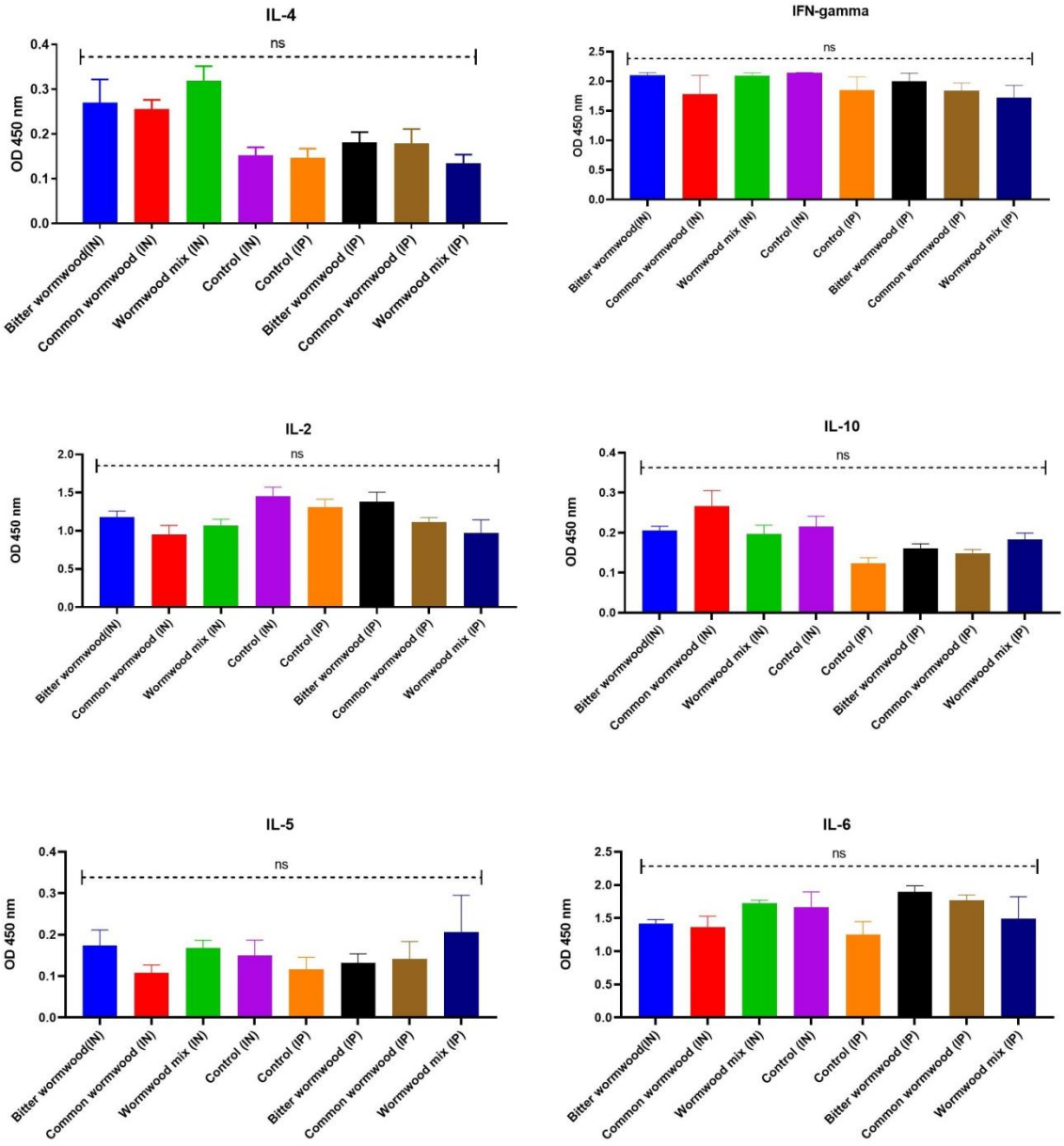
It was found (Figure 5A) that in all groups of mice after challenge, including control mice, to which a PBS was administered intranasally, such signs as nose rubbing were observed (and repeated rubbing was counted as one), ear scratching. The greatest manifestation of these signs was noted in the experimental groups, but they did not have a statistically significant difference from those of the corresponding control groups. It is noteworthy that exclusively in the group of mice sensitized by the IN regime with bitter wormwood extract, a sign with biting of their own tail was noted, which was also observed during the process of sensitization. At the same time, the tails were covered with numerous hemorrhages (Figure 6).

The allergy ear swelling test (Figure 5B) showed the presence of a pronounced allergy after challenge in groups of mice with IP sensitization, where extracts of bitter and common wormwood were used (P=0.0023-<0.0001 for the corresponding control group). At the same time, the level of auricular edema in the group of animals with bitter wormwood was significantly higher (P=0.044-<0.0001) than in other experimental groups.

*Assessment of inflammatory and destructive changes in the lungs of mice after allergic challenge*

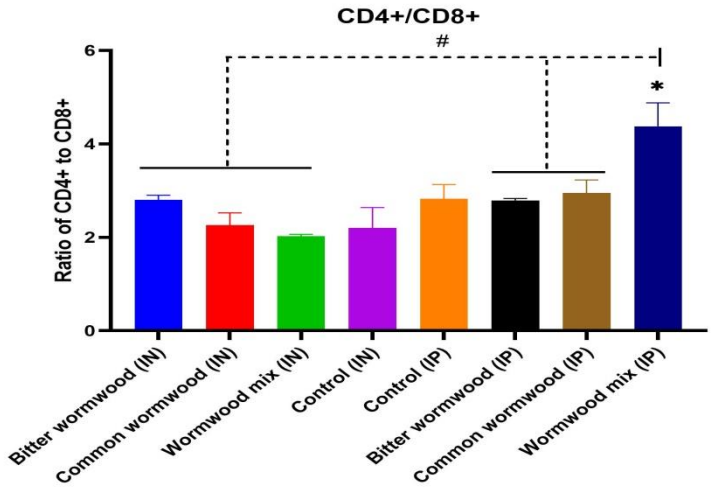
For this purpose, lungs were harvested from mice of all groups after euthanasia, some of which were fixed with 10% formaldehyde for histological analysis, and another part was homogenized in RPMI to measure proinflammatory cytokines (IL-5, IL-6).

Histological analysis of the lungs (Figure 7) showed the presence of perivascular inflammation in the lungs of mice from all experimental groups, but it was most pronounced (P=0.034-0.0061 vs. corresponding controls) in the groups with IP sensitization using wormwood extracts and wormwood mix, as well as in one group with IN sensitization (wormwood mix). As for the number of eosinophils in the lungs responsible for allergic inflammation, their significant (P<0.0001) accumulation compared with the corresponding controls was observed in all experimental groups (Figure 7). However, the highest number of eosinophils was observed with the IP regime of sensitization using common wormwood, which, moreover, was significantly higher (P<0.0001) in comparison with the IN regime of sensitization.



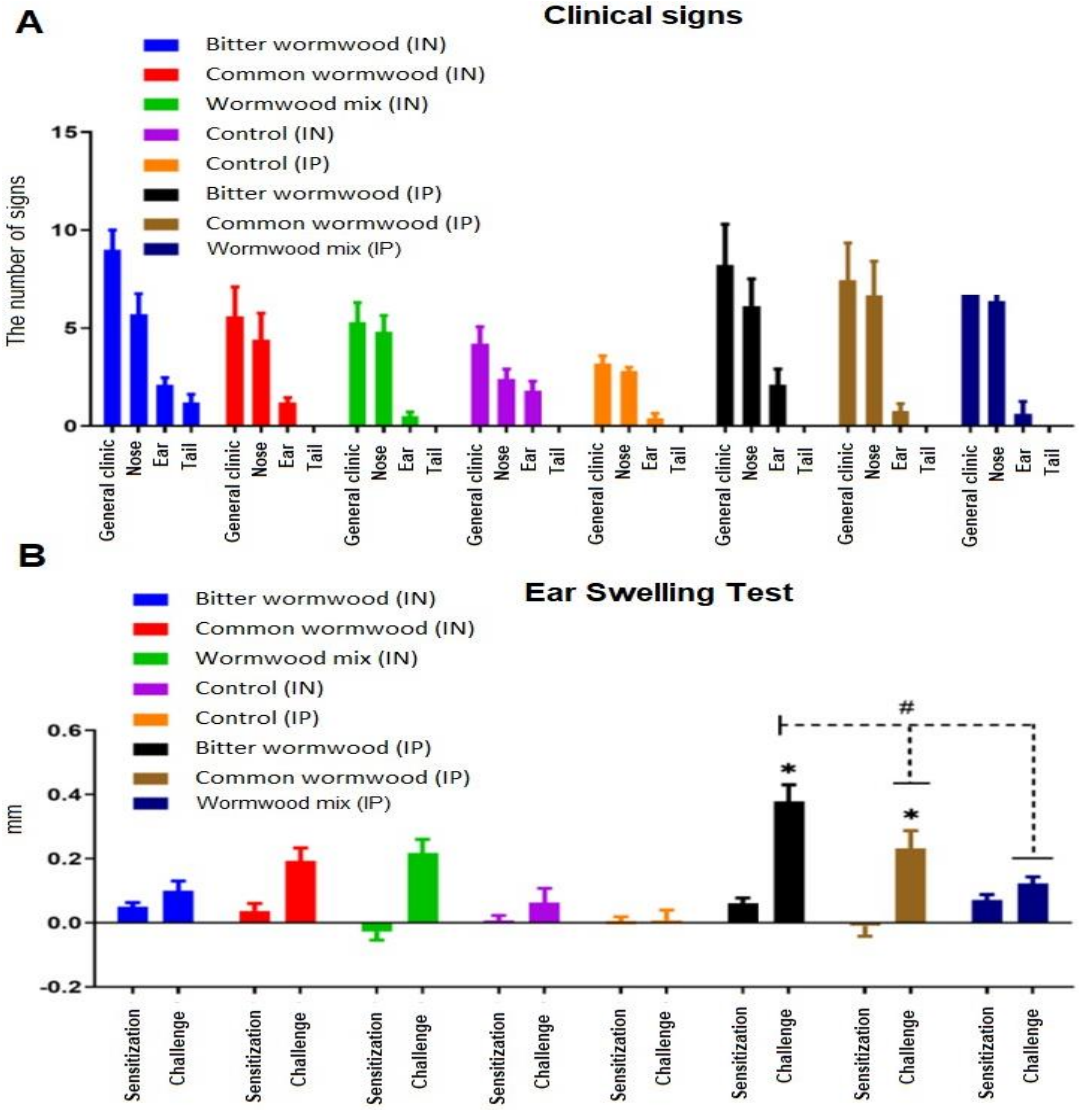

NS - not a significant difference

**Figure 3. rArt V1 protein-induced cytokine profile in SPF BALB/c mice after challenge with wormwood extracts.**




IN - intranasal; IP - intraperitoneal, (*) P = 0.041 to the corresponding control group; # P = 0.034-0.0005

**Figure 4. rArt V1 protein-induced CD4+/CD8+ cell immunogram in a suspension of mice splenocytes after challenge with wormwood extracts.**




IN - intranasal; IP- intraperitoneal, (*) P = 0.0023-<0.0001 to the corresponding control group; # P = 0.044-<0.0001.
(A) – clinical signs of allergic reactions (rubbing the nose, scratching the ear where the allergen was injected, biting the tail);
(B) – ear swelling test.

**Figure 5. Clinical signs of allergic reactions and ear swelling test in SPF BALB/c mice after sensitization and challenge with wormwood extracts.**

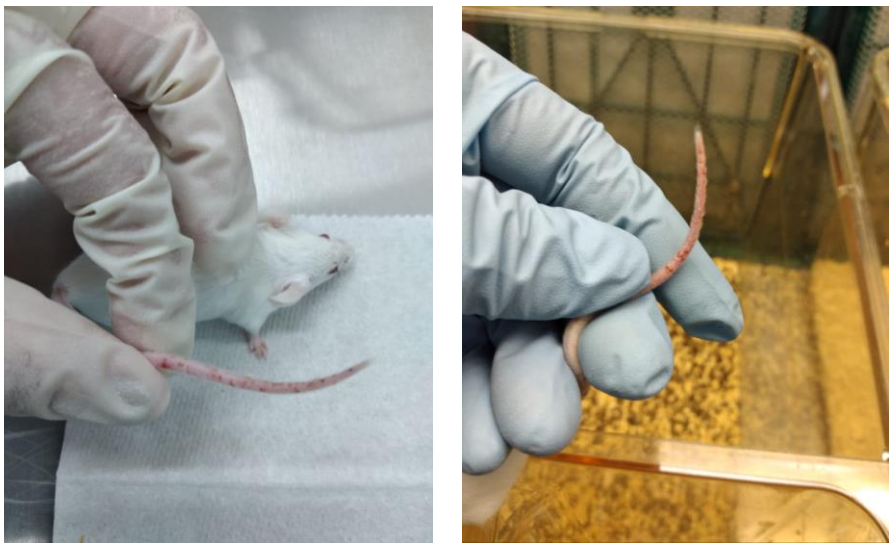
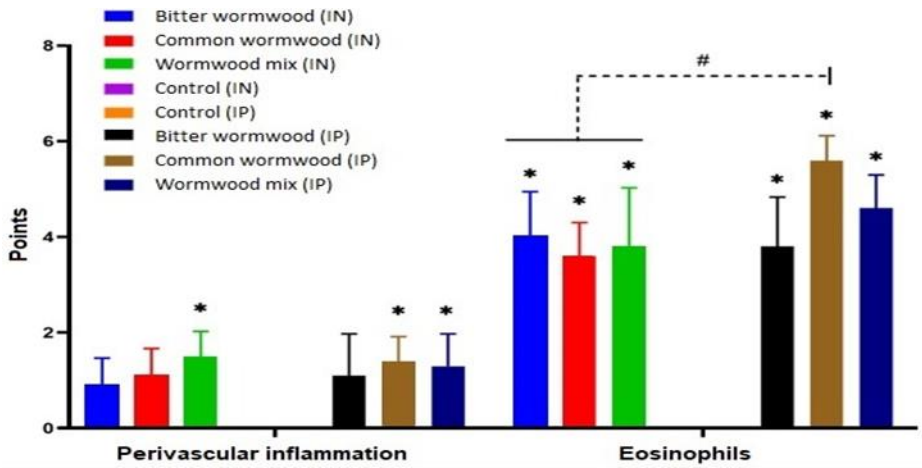


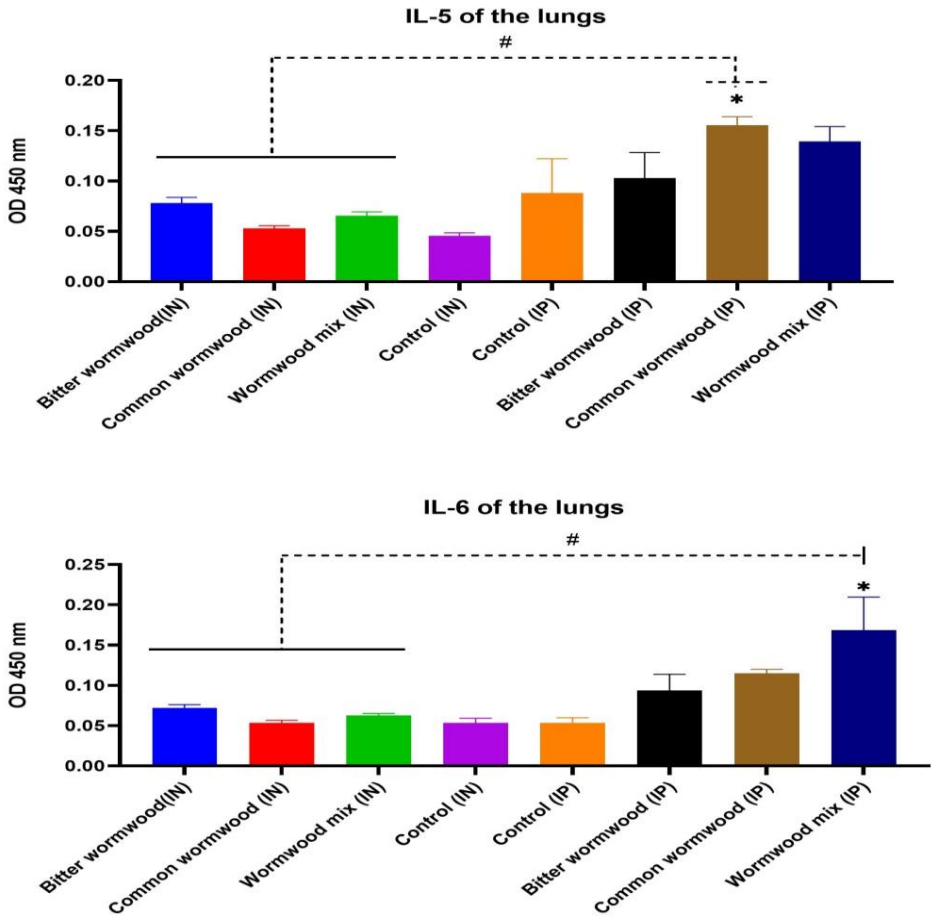
**Figure 6. View of mice tails as a result of biting.**




IN - intranasal; IP - intraperitoneal, (*) P = 0.034-<0.0001 to the corresponding control group; # P <0.0001.
The extent of perivascular inflammation was expressed by the number of crosses (from 0 to 4),
which were translated into appropriate numbers for analysis.

**Figure 7. Histological analysis of the lungs of SPF BALB/c mice after challenge with wormwood extracts.**

Evaluation of proinflammatory cytokines (Figure 8) in mouse lung homogenates showed that significant (P=0.022 vs. corresponding control) expression of cytokine IL-5 (responsible for allergic inflammation) was noted in mice of the group with IP sensitization regime, where wormwood extract was used. Moreover, this index was significantly (P=0.0005-<0.0001) superior to the data in the groups with the IP sensitization regime. For the general inflammatory cytokine IL-6, the highest (P=0.003 versus the corresponding control; P=0.0017-0.0002 versus the groups with IN sensitization) data were demonstrated by the group with IP sensitization regime when using an extract from the wormwood mix.




IN - intranasal; IP - intraperitoneal, (*) P = 0.026-0.003 0001 to the corresponding control group; # P = 0.0041-<0.0001.

**Figure 8. Assessment of allergic proinflammatory cytokine levels in lung homogenates of SPF BALB/c mice after challenge with wormwood extracts.**

**Discussion of Results**

In this study, we carried out the reproduction and testing of a mouse model of allergic rhinitis and bronchial asthma. As previously noted, for the sensitization of BALB/c mice, we used the two most common species of wormwood and their mix responsible for the largest cases of seasonal pollinosis in Kazakhstan.

It should be noted that this work was the first time that wormwood extracts were used for sentinization of mice, and therefore the results obtained are unique and cannot be compared with other works.

When assessing the level of allergization of mice depending on the method of sensitization and the type of allergen, we used a comprehensive approach that included the analysis of antibody (general and antigen-specific IgE, IgG1, IgG2a, IgA antibodies), cytokine, cellular immune responses, clinical signs, ear swelling test and histological analysis of the lungs. In the course of these studies, we did not receive absolutely unambiguous and comparable data on all the parameters studied in relation to any one group. For example, in a group of mice with instigation, when using bitter wormwood extract, we observed an interesting manifestation of an allergic reaction in the form of tail biting (Figure 6). However, at the same time, these mice lacked common and antigen-specific allergic IgE antibodies, the results of the ear swelling test were negative, no destructive changes in the lungs were observed. It was obvious that the IP regime of sensitization was more effective than the IN regime. At the same time, among the tested allergens, the most allergenic was the extract of common wormwood, which, in the IP regime of sensitization in mice:

1) formed high values of total and antigen-specific IgE antibodies;
2) by the ratio of IgG1/IgG2a antibodies induced a pronounced polarization of the immune response toward Th2;
3) reacted positively in the ear swelling test;
4) induced perivascular and eosinophilic inflammation in the lungs with expression of the allergic proinflammatory cytokine IL-5.

Reliability of the obtained data was ensured by the number of sufficient animal samples (n=10 in the experimental, n=5 in the control groups), as well as by the methods of adequate statistical processing of the research results. The level of research design and depth allowed to obtain new experimental data with good publication opportunities.

**Conclusion**

To model on mice allergic rhinitis and bronchial asthma caused by wormwood pollen, it is more effective to use the extract of common wormwood with IP sensitization regime.

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