

Article

Effects of a Nasal Spray Based on an Antigen Complex from Opportunistic Bacteria in Experimental Models of SARS-CoV-2 and Influenza A Infection

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Abstract

Background/Objectives: Acute respiratory infections represent a global health burden due to their high incidence, morbidity and mortality. Despite advances in prevention and treatment, strategies providing broad protection against respiratory pathogens remain limited. This study evaluated the antiviral activity of nasal spray forms based on an antigen complex from opportunistic bacteria, with or without a mucoadhesive copolymer, and their influence on cellular and humoral components of the immune response. **Methods:** Antiviral activity was assessed in experimental models of SARS-CoV-2 infection in Syrian hamsters and influenza A pneumonia in mice under prophylactic and therapeutic–prophylactic regimens. Parameters of cellular and humoral immune response were assessed using delayed-type hypersensitivity, antibody-forming cell assays, and leukocyte phagocytic activity. **Results:** Both forms showed comparable influence on cellular and humoral components of the immune response. In a mouse model of lethal influenza pneumonia induced by A/California/04/2009 (H1N1)pdm09 virus, intranasal administration at 100 µg/kg increased mean survival time and survival rate, and reduced body weight loss. In SARS-CoV-2-infected hamsters, both forms reduced clinical signs, body weight loss, weight lung index, lung pathology, and decreased viral titers. The copolymer-containing form showed the most pronounced effect under the therapeutic–prophylactic regimen, with greater protection against body weight loss and stronger suppression of viral replication. **Conclusions:** The findings support further investigation of these nasal spray forms as candidates for prophylaxis and adjunctive therapy of respiratory infections, particularly when pathogen variability may limit the effectiveness of pathogen-directed treatments. Further mechanistic studies are needed to clarify the pathways underlying the observed antiviral effect.

Keywords: bacterial lysates; respiratory disease; influenza; SARS-CoV-2; Syrian hamsters

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1. Introduction

Acute respiratory infections remain the most significant infectious diseases world-wide due to their high prevalence and substantial morbidity and mortality rates. Despite considerable advances in prevention and treatment, respiratory diseases continue to represent a major global health challenge [1–4].

Acute respiratory infections are characterized by a multifactorial etiology and can be caused by viral, bacterial, and fungal pathogens, as well as their combinations [5–7]. Viruses, including coronaviruses, influenza viruses, and other respiratory viruses, play a predominant role in upper respiratory tract infections [8]. Bacterial pathogens frequently contribute to coinfections, exacerbating disease severity and increasing the risk of complications [9,10]. Interactions among multiple microorganisms lead to impairment of the respiratory epithelial barrier, enhanced inflammatory responses, and immune dysfunction, which are particularly pronounced in immunocompromised individuals [11–13].

The broad spectrum of respiratory pathogens and the high incidence of coinfections highlight the need for therapeutic strategies targeting host innate defense mechanisms rather than individual pathogens. In this context, preparations containing antigens of opportunistic bacteria have attracted considerable interest due to their ability to activate innate immune responses [14–17]. Bacterial lysates are widely used in the prevention and treatment of respiratory tract infections and have been shown to enhance host resistance to both bacterial and viral infections [18–21].

Despite the diversity of available bacterial lysates [22–24], certain features of their dosage forms and dosing regimens may limit their use in clinical practice. This highlights the importance of developing new dosage forms aimed at simplifying dosing regimens and improving bioavailability. A promising strategy involves the development of intranasal drug forms that directly target the respiratory mucosa and activate mucosal immune responses. These forms may combine efficacy with ease of administration, making them useful in approaches aimed at reducing the burden of respiratory infections [25–27].

As part of the development of intranasal immunobiological agents, a nasal spray based on a complex of antigens of opportunistic bacteria, including *Proteus vulgaris*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Escherichia coli*, is being developed at the I. I. Mechnikov Research Institute for Vaccines and Sera (Moscow, Russia). The nasal spray was studied in two forms, with or without the mucoadhesive 2-methyl-5-vinylpyridine/N-vinylpyrrolidone hydrochloride copolymer, to determine how prolonged contact with the mucosa influences the immune response [28,29].

The aim of the present study was to assess the effects of two nasal spray forms based on an antigen complex from opportunistic bacteria on cellular and humoral components of the immune response, as well as their antiviral activity in experimental models of SARS-CoV-2 infection in Syrian hamsters and influenza-induced pneumonia in mice.

2. Materials and Methods

2.1. Pathogens and Cell Lines

Phagocytic activity was evaluated using the *Staphylococcus aureus* Wood 46 strain obtained from the Collection of Microorganisms of Pathogenicity Groups III–IV, Mechnikov Research Institute of Vaccines and Sera (Moscow, Russia).

Vero CCL81 cells were obtained from the American Type Culture Collection (ATCC). The cells were maintained in DMEM (PanEko, Moscow, Russia) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine (PanEko, Moscow, Russia), 100 U/mL penicillin, and 100 µg/mL streptomycin (PanEko, Moscow, Russia). Cultures were maintained at 37 °C in a humidified 5% CO₂ atmosphere.

The SARS-CoV-2 ‘Dubrovka’ strain (GenBank: MW161041.1), isolated from a nasopharyngeal swab of a patient with confirmed COVID-19 and propagated in Vero CCL81 cells, was used to evaluate the antiviral activity of the nasal spray forms. The virus was cultured at 37 °C in DMEM supplemented with 4.5 g/L glucose (Sigma-Aldrich, St. Louis, MO, USA), 5% FBS, 300 µg/mL L-glutamine, and 40 µg/mL gentamicin under 5% CO₂. The strain underwent 17 consecutive passages and induced pronounced cytopathic effects. Viral samples for the study were stored in aliquots at −80 °C.

To evaluate the efficacy of the nasal spray forms against influenza virus, MDCK cells (Madin Darby Canine Kidney) obtained from the collection of the N.F. Gamaleya National Research Center for Epidemiology and Microbiology (Moscow, Russia) were used. MDCK cells were maintained in MEM (CellGro, Manassas, VA, USA) containing 10% FBS and 300 µg/mL L-glutamine. The medium was additionally supplemented with antibiotics: 100 µg/mL penicillin, 100 µg/mL streptomycin sulfate, and 100 µg/mL kanamycin sulfate. Cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere.

For the mouse infection model, influenza virus A/California/04/2009 (H1N1) pdm09 was provided by the WHO National Influenza Centre of Russia (St. Petersburg, Russia) and mouse-adapted by three lung-to-lung passages. Aliquots were stored at −80 °C, and the virus titer was 10⁷ TCID₅₀.

2.2. Animals

The effects of the nasal spray forms on cellular and humoral components of the immune response were assessed in CBA and C57BL/6 mice obtained from the Stolbovaya branch of the NCBMT FMBA Institution (Moscow, Russia). The study included 360 CBA mice (180 males and 180 females) and 60 C57BL/6 mice (30 males and 30 females, 20–22 g). C57BL/6 mice were used to assess phagocytic activity, whereas CBA mice were used to evaluate delayed-type hypersensitivity responses (120 animals) and antibody-forming cell (AFC) numbers (240 animals).

The efficacy of the nasal spray forms against SARS-CoV-2-induced infection was evaluated in 48 female Syrian golden hamsters weighing 50–60 g, obtained from the Stezar nursery (Vladimir, Russia). Their efficacy against influenza virus infection was evaluated in 90 female BALB/c mice weighing 12–14 g, obtained from the Andreevka branch of the NCBMT FMBA Institution (Moscow, Russia).

Animals had free access to food and water throughout the study, and environmental enrichment was provided. All procedures involving animals were performed in accordance with FELASA guidelines [30] and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, France, 18 March 1986). The study protocol was reviewed and approved by the Research Ethics Committee of the I. I. Mechnikov Research Institute for Vaccines and Sera, Moscow, Russia (approval No. 14, 3 December 2024).

2.3. Compounds

The nasal spray contained an antigen complex prepared from water-soluble extracts of microbial cells of four opportunistic bacterial species: *K. pneumoniae*, *E. coli*, *P. vulgaris*, and *S. aureus*. The bacterial components were included in equal mass proportions. The base nasal spray contained the antigen complex at 1.0 mg/mL, lactose at 25.0 mg/mL, disodium hydrogen phosphate at 0.20 mg/mL, sodium chloride at 0.175 mg/mL, potassium dihydrogen phosphate at 0.02 mg/mL, and purified water up to 1 mL.

This base nasal spray was evaluated in two forms: without a mucoadhesive component and with 2-methyl-5-vinylpyridine/N-vinylpyrrolidone hydrochloride at 1.0 mg/mL. The copolymer had a molecular weight of 35–55 kDa, with monomer unit contents of $n = 25$ –90 mol% and $m = 0.1$ –1.0 mol%. The copolymer was included to reduce washout of the

antigen complex from the nasal mucosa. Its mucoadhesive properties were confirmed in adhesion tests using model substrates, including a mucin film. Direct measurement of residence time on the nasal mucosa was not performed [28].

2.4. Experimental Design

Two series of experiments were conducted:

1. Assessment of the influence of the nasal spray forms on cellular and humoral components of the immune response, including phagocytic activity as an indicator of innate cellular immunity, delayed-type hypersensitivity as an indicator of cellular immune response, and the number of antibody-forming cells (AFCs) in the spleen as an indicator of humoral immune response.
2. Assessment of antiviral activity in experimental models of SARS-CoV-2 and influenza virus infection.

2.5. Phagocytic Activity

To study the effect of the nasal spray forms on phagocytic activity, C57BL/6 mice were divided into five groups: four experimental groups, each consisting of 5 females and 5 males, and a control group of 10 females and 10 males. Animals were allocated to experimental groups according to their individual body weight, with deviations not exceeding 10% from the mean weight of animals in each experiment (Table 1). Intranasal administration in the immune response assays was performed dropwise using a calibrated automatic micropipette at a volume of 5 µL per nostril. Working dilutions were prepared according to body weight to ensure delivery of the required dose of the antigen complex.

Table 1. Experimental design for the assessment of phagocytic activity.

Group	Test Sample/Dose	Regimen
1	With copolymer (3 µg/kg)	Days 1–10—intranasal administration of the test sample (5 µL per nostril), once daily
2	Without copolymer (3 µg/kg)	
3	With copolymer (0.5 mg/kg)	Day 11—intraperitoneally 3–4 mL of 2–3% peptone solution
4	Without copolymer (0.5 mg/kg)	
5	Control group (physiological saline)	

On day 11, 2 h after the administration of the peptone solution, mice were euthanized by cervical dislocation. Then, fluid was aspirated from the peritoneal cavity, transferred into siliconized centrifuge tubes, and centrifuged for 10 min at 1000 rpm. The pellet was resuspended in Medium 199 (PanEko, Moscow, Russia), the cell count was determined using a Goryaev chamber, and the neutrophil concentration was adjusted to $1\text{--}2 \times 10^6$ cells/mL.

S. aureus Wood 46 was cultured on meat-peptone agar (PanEko, Moscow, Russia), then washed off with 0.9% NaCl solution, heat-killed at 96–98 °C for 40 min, and centrifuged at 3000× g for 10 min. The bacterial pellet was washed twice with 10 mL of phosphate-buffered saline (PBS), resuspended in carbonate–bicarbonate buffer (pH 9.5) to a final concentration of 1×10^9 cells/mL, and labeled with fluorescein isothiocyanate (FITC) at a final concentration of 0.1 mg/mL. The suspension was incubated at 4 °C for 12 h. Unbound FITC was then removed by three washes with PBS followed by centrifugation at 3000× g for 10 min.

To 100 µL of the peritoneal leukocyte suspension, 100 µL of FITC-labeled *S. aureus* (at a concentration of 10^9 microbial cells/µL) was added and incubated for 30 min at 37 °C. Then, 100 µL of OptiLyse C lysis solution (Beckman Coulter, Brea, CA, USA) was added to lyse erythrocytes and fix the cells, followed by incubation for 15 min at room temperature in the dark. Unbound bacteria and debris were removed by washing three times with

tenfold volumes of PBS, centrifuging at 1000 rpm for 3 min. The washed leukocytes were resuspended in 500 µL of PBS. The number of leukocytes that had phagocytosed the FITC-labeled bacteria was determined using a Cytomics FC-500 flow cytometer (Beckman Coulter, Fullerton, CA, USA).

The cell population gate was determined by assessing forward and side scatter (cell size and granularity); 10,000 cells were analyzed within the gate. Cells exhibiting fluorescence in the FL1 channel above the threshold (10^2) were considered to have phagocytosed bacteria. The results were expressed as the percentage of leukocytes that had phagocytosed heat-killed FITC-labeled *S. aureus* cells.

2.6. Delayed-Type Hypersensitivity Reaction

To assess DTH, mice were divided into 12 groups of 10 animals each (5 females and 5 males per group) (Table 2).

Table 2. Experimental design for evaluating the effects of nasal spray forms on the cellular immune response.

Group	Test Sample/Dose	Regimen
1	With copolymer (3 µg/kg)	Days 1–9—intranasal administration of the test sample (5 µL per nostril), once daily. Day 10—intranasal administration of the test sample (5 µL per nostril) followed by a sensitizing dose of sheep erythrocytes (1×10^7 cells per mouse) administered in a volume of 100 µL at the base of the tail. Day 15—provocative dose of sheep erythrocytes (1×10^8 cells per mouse) administered in a volume of 50 µL into the plantar surface of the hind paw; 50 µL of physiological saline was injected into the contralateral “control” paw.
2	Without copolymer (3 µg/kg)	
3	With copolymer (0.5 mg/kg)	
4	Without copolymer (0.5 mg/kg)	
5	Control group (physiological saline)	
6	Intact group	Days 1–9—intranasal administration of physiological saline (5 µL per nostril), once daily. Day 10—intranasal administration of physiological saline (5 µL per nostril) followed by injection of physiological saline (100 µL) at the base of the tail. Day 15—provocative dose of sheep erythrocytes (1×10^8 cells per mouse) administered in a volume of 50 µL into the plantar surface of the hind paw; 50 µL of physiological saline was injected into the contralateral “control” paw.
7	With copolymer (3 µg/kg)	Day 1—sensitizing dose of sheep erythrocytes (1×10^7 cells per mouse) administered in a volume of 100 µL at the base of the tail. Day 5—intranasal administration of the test sample (5 µL per nostril) followed by a provocative dose of sheep erythrocytes (1×10^8 cells per mouse) injected in a volume of 50 µL into the plantar surface of the hind paw; 50 µL of physiological saline was injected into the contralateral “control” paw.
8	Without copolymer (3 µg/kg)	
9	With copolymer (0.5 mg/kg)	
10	Without copolymer (0.5 mg/kg)	
11	Control group (physiological saline)	
12	Intact group	Day 1—physiological saline (100 µL) was injected at the base of the tail. Day 5—intranasal administration of physiological saline (5 µL per nostril) was performed, followed by a provocative dose of sheep erythrocytes (1×10^8 cells per mouse) injected in a volume of 50 µL into the plantar surface of the hind paw; 50 µL of physiological saline was injected into the contralateral “control” paw.

The DTH response was evaluated 24 h after administration of the provocative dose of sheep erythrocytes. Mice were euthanized by cervical dislocation, after which the hind paws were excised and weighed. Experimental and control paws were excised at the same anatomical level, along the ankle joint line, by the same trained operator according to a standardized protocol. The paws were then dried in an incubator for 48 h at 39 °C and weighed again.

Based on the weighing results, two parameters were calculated: the reaction index based on paw mass ratio and the reaction index based on paw fluid content.

The reaction index based on paw mass ratio was calculated for each animal using the wet mass of the paws before drying according to the following formula: Reaction index based on paw mass ratio (Rim, %) = $[(m_e - m_c) / m_c] \times 100$, where m_e is the mass of the experimental paw before drying, and m_c is the mass of the control paw before drying.

Fluid content was calculated separately for the experimental and control paws as follows: Fluid content (FC, %) = $[(m_{\text{before}} - m_{\text{after}}) / m_{\text{before}}] \times 100$, where m_{before} is the paw mass before drying, and m_{after} is the paw mass after drying.

The reaction index based on paw fluid content was calculated as follows: Reaction index based on paw fluid content (Rif, %) = $[(FC_e - FC_c) / FC_c] \times 100$, where FC_e is the fluid content in the experimental paw, and FC_c is the fluid content in the control paw.

2.7. Assessment of Antibody-Forming Cells in Response to Nasal Spray Forms

The effect of the nasal spray forms on the humoral immune response was evaluated by determining the number of AFC using the Jerne–Nordin local hemolysis assay.

For the assay, spleens were harvested and splenocyte suspensions were prepared using a glass homogenizer. The suspensions were filtered through a double nylon mesh, and after counting in a Goryaev chamber, splenocyte concentrations were adjusted to 1×10^7 cells/mL. A mixture consisting of 2% agarose melted in Hanks' solution, splenocytes, and sheep erythrocytes was prepared. Specifically, 300 µL of splenocyte suspension and a three-times-washed pellet of sheep erythrocytes were added to 3 mL of molten agarose maintained at 45 °C in a water bath. The mixture was poured into plastic Petri dishes and evenly distributed over the surface. After agar solidification, the plates were incubated at 37 °C for 1 h. Guinea pig serum complement diluted 1:10 was then added, followed by an additional 1 h incubation at the same temperature. Results were evaluated visually by counting hemolytic zones (clear plaques) against a red background, each corresponding to a single IgM-producing AFC. The number of AFC was calculated per 10^6 nucleated cells and per whole spleen.

To assess the effect of the nasal spray forms on AFC numbers, 240 mice were allocated to 12 groups, each consisting of 10 females and 10 males (Table 3).

Table 3. Experimental design for the assessment of antibody-forming cells.

Group	Test Sample/Dose	Regimen
1	With copolymer (3 µg/kg)	Days 1–9—intranasal administration of the test sample (5 µL per nostril) once daily.
2	Without copolymer (3 µg/kg)	
3	With copolymer (0.5 mg/kg)	Day 10—intranasal administration of the test sample (5 µL per nostril) followed by intraperitoneal injection of sheep erythrocytes at a dose of 5×10^6 cells per mouse in a volume of 200 µL.
4	Without copolymer (0.5 mg/kg)	
5	Control group (physiological saline)	Antibody-forming cells were assessed 3 days after the final immunization.
6	Intact group	Days 1–9—intranasal administration of physiological saline (5 µL per nostril) once daily.

		Day 10—intranasal administration of physiological saline (5 µL per nostril) followed by intraperitoneal injection of physiological saline in a volume of 200 µL. Antibody-forming cells were assessed 3 days after the final immunization.
7	With copolymer (3 µg/kg)	Day 1—intraperitoneal injection of sheep erythrocytes at a dose of 5×10^6 cells per mouse in a volume of 200 µL. Day 5—intranasal administration of the test sample. Antibody-forming cells were assessed 5 days after immunization.
8	Without copolymer (3 µg/kg)	
9	With copolymer (0.5 mg/kg)	
10	Without copolymer (0.5 mg/kg)	
11	Control group (physiological saline)	
12	Intact group	Day 1—intraperitoneal injection of physiological saline in a volume of 200 µL. Day 5—intranasal administration of physiological saline (5 µL per nostril). Antibody-forming cells were assessed 5 days after immunization.

2.8. Assessment of Antiviral Activity

Antiviral activity was assessed using the same six-group experimental design for both infection models. The SARS-CoV-2 infection model was performed in female Syrian golden hamsters (6 animals per group), whereas the influenza virus infection model was performed in female BALB/c mice (15 animals per group) (Table 4).

Table 4. Animal group distribution in antiviral activity assessment.

Group	Test Sample/Dose	Regimen
1	Without copolymer, 100 µg/kg	Infected animals that received the nasal spray forms intranasally for 5 days prior to infection, with the final dose administered 1 h before infection (prophylactic regimen).
2	With copolymer, 100 µg/kg	
3	Without copolymer, 100 µg/kg	Infected animals received the nasal spray forms intranasally for 3 days prior to infection, 1 h after being infected on the treatment day, and for 3 consecutive days post-infection (therapeutic–prophylactic regimen).
4	With copolymer, 100 µg/kg	
5	Virus control	Infected animals received intranasal administration of distilled water for 5 days prior to infection, with the final administration given 1 h before infection.
6	Intact group	Non-infected animals received intranasal administration of distilled water according to the same schedule.

The test samples were administered intranasally using a calibrated automatic micropipette. The single administration volume was 100 µL for hamsters (50 µL per nostril) and 50 µL for mice (25 µL per nostril). Before administration, working dilutions of the forms were prepared according to the body weight of the animals to ensure that the fixed administered volume contained the required dose of the antigen complex. Animals in Group 5 (Viral control) and Group 6 (Intact group) received distilled water in equivalent volumes.

2.9. SARS-CoV-2 Infection Model

To evaluate the efficacy of the nasal spray forms in the SARS-CoV-2 infection model, all animals in Groups 1–5 were intranasally infected with the SARS-CoV-2 “Dubrovka” strain after light ether anesthesia, with a total volume of 100 µL (50 µL per nostril). The infection dose was 10^5 TCID₅₀/mL.

Animals were monitored daily throughout the duration of the experiment. The efficacy of the nasal spray forms in the infection model was assessed based on body weight

dynamics, weight lung index (WLI), viral titers in the lungs, and pathological changes in lung tissues. Female Syrian golden hamsters were weighed daily prior to administration of the test forms. Weight loss or gain was calculated for each animal and expressed as a percentage, with 100% corresponding to the body weight prior to infection. The mean percentage of weight loss or gain was determined for each group.

On days 3 and 6 post-infection, three animals from each group were euthanized by cervical dislocation after induction of anesthesia with isoflurane. Lungs were aseptically collected for subsequent analyses. After a visual pathological assessment, lungs were weighed to determine the WLI. The right lung was washed three times with 0.01 M PBS and fixed in formalin for histological examination. Lung tissue sections were coded before evaluation. Histopathological and morphometric assessment of lung tissue sections was performed by an observer blinded to group allocation. The left lung was placed in 1 mL of cold sterile PBS and homogenized. After centrifugation at $2000\times g$ for 10 min to remove cellular debris, the supernatant was collected for determination of the infectious virus titer in cell culture.

Lungs collected aseptically were weighed to determine the WLI, which was calculated for each animal using the following formula: $WLI = (\text{weight of the lung, g} / \text{animal weight, g}) \times 1000$.

The mean WLI was then calculated for all hamsters within each group.

To measure infectious virus titers in hamster lung samples, Vero CCL81 cells were plated in 96-well Costar plates at 20,000 cells per well and maintained in DMEM containing 5% fetal calf serum, 10 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin until a complete monolayer had formed. Prior to infection, the cell culture was washed twice with serum-free DMEM. Tenfold serial dilutions of each lung virus sample (from 10^{-1} to 10^{-7}) were prepared. Aliquots of 200 µL from each dilution were added to the cell culture plates and incubated at 37 °C in a 5% CO₂ atmosphere for 5 days until cytopathic effects appeared in the virus control wells. Viral titers were quantified by the MTT assay and calculated using the method described by Ramakrishnan [31]. Results were reported as log₁₀ TCID₅₀/0.1 mL. Group mean titers were calculated from the individual lung samples of hamsters in each group. A reduction in viral titer of ≥ 1.5 log₁₀ TCID₅₀/0.1 mL compared to the virus control was considered significant.

2.10. Mouse Model of Influenza Infection

Pre-weighed mice were intranasally infected with influenza virus A/California/04/2009 (H1N1)pdm09 at a dose of 5 MLD₅₀. The virus was administered as 15 µL of viral suspension per nostril after induction of anesthesia with isoflurane.

The efficacy of the nasal spray forms in the mouse model of influenza infection was assessed based on the following parameters: animal survival, mean survival time (MST), body weight dynamics, and viral titers in the lungs on day 4 post-infection.

The percentage of mortality was calculated as the ratio of the number of animals that died to the total number of infected animals in the group. The MST was calculated using the following formula: $MST = \sum f(d - 1) / n$, where f is the number of mice that died on day d (surviving mice are also included, with $d = 14$ for them), and n is the total number of mice in the group.

Each group included 15 animals, of which 5 were used to analyze viral titers in the lungs, and the remaining 10 were monitored for survival parameters. Mice were weighed on the day of infection (day 0) and on days 2, 4, 6, 8, 10, 12, and 14 post-infection. Weight loss or gain was calculated individually for each mouse and expressed as a percentage.

On day 4 post-infection with influenza virus, five mice from each group were humanely euthanized. Aseptically collected lungs were homogenized in 1 mL of cold sterile 0.01 M PBS. Cellular debris was removed by centrifugation at $2000\times g$ for 10 min. An

aliquot of 0.1 mL of the supernatant was used to determine the infectious virus titer in MDCK cell culture.

To determine the infectious virus titer, MDCK cells were seeded in 96-well Costar plates at an average density of 30,000–35,000 cells per well and cultured in MEM supplemented with 5% fetal calf serum, 10 mM L-glutamine, and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) until a complete monolayer was formed. Prior to infection, the cell culture was washed twice with serum-free MEM. Tenfold serial dilutions of each lung virus sample (up to 10^{-9}) were prepared in medium containing 2 µg/mL trypsin-TPCK. Each dilution was applied to quadruplicate wells of the 96-well monolayer. After incubation at 37 °C in a 5% CO₂ atmosphere for 72 h, the cells were washed three times with PBS and fixed with 10% formaldehyde at 18–23 °C for 5 min. After removal of the formaldehyde solution, 100 µL of 1% crystal violet solution was added to each well and incubated at 18–23 °C for 5 min. Plates were then rinsed with water, allowed to dry, and 0.1 mL of 96% ethanol was added per well, followed by incubation with gentle shaking at room temperature for 20 min. Optical density was measured at 570 nm. Wells were considered “positive” if the optical density was at least 20% lower than that of the cell control. Infectious virus titers were calculated using the Reed and Muench method in quadruplicate for each sample from an individual animal and expressed as log₁₀ TCID₅₀/0.1 mL. Mean titers were calculated from five individual samples per group.

The primary criterion for antiviral efficacy was an increase in animal survival, with a protection index (PI) of at least 60%. Secondary criteria included reduced body weight loss, reflecting lower disease severity, and a ≥ 2 log₁₀ TCID₅₀/0.1 mL reduction in lung viral titers compared with the virus control group.

2.11. Data Analysis

Statistical analysis was performed using Python (version 3.12.7) with the following libraries: NumPy (version 1.26.4), pandas (version 2.3.3), SciPy (version 1.13.1), statsmodels (version 0.14.2), matplotlib (version 3.9.2), and seaborn (version 0.13.2).

For immune-response experiments, including phagocytic activity, delayed-type hypersensitivity, and antibody-forming cell counts, data are presented as mean $\pm$ standard error of the mean (SEM). Differences between groups were analyzed using the two-sided Mann–Whitney U test.

For infection experiments, data are presented as mean $\pm$ standard deviation (SD). Quantitative endpoints were analyzed separately for each model and, where applicable, for each time point. Viral titers, WLI, and endpoint body-weight change were compared among infected groups using the Kruskal–Wallis test followed by Dunn’s post hoc test against the viral control group with Bonferroni correction for multiple comparisons. Viral titers were analyzed on the log₁₀ scale. Body weight change was calculated for each animal as the percentage change from baseline body weight before virus inoculation. Survival was analyzed using Kaplan–Meier estimates and the log-rank test with Bonferroni adjustment for pairwise comparisons with the viral control group.

Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effects of the Nasal Spray Forms on Phagocytic Activity

The highest phagocytic activity was observed in the control group. In animals receiving the tested nasal spray forms for 10 days, a statistically significant decrease in phagocytic activity was observed relative to the control ($p < 0.05$). Moreover, a pronounced and statistically significant dose-dependent effect was identified ($p < 0.05$): animals treated with the higher dose exhibited greater phagocytic activity. No statistically significant

differences were found between different nasal spray forms administered at the same doses (Figure 1).

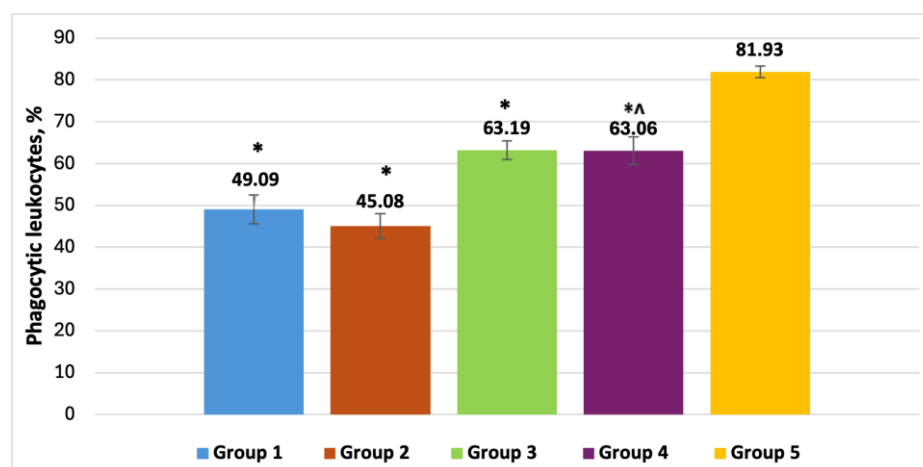


Figure 1. Mean phagocytic activity of leukocytes. Group 1, nasal spray with copolymer (3 µg/kg); Group 2, nasal spray without copolymer (3 µg/kg); Group 3, nasal spray with copolymer (0.5 mg/kg); Group 4, nasal spray without copolymer (0.5 mg/kg); Group 5, control group (physiological saline); *— $p < 0.05$ versus the control group; ^— $p < 0.05$ versus animals receiving the same nasal spray form at a lower dose.

3.2. Effects of the Nasal Spray Forms on the Cellular Immune Response in the Delayed-Type Hypersensitivity Reaction

Analysis of the data obtained after 10 days of administration of the nasal spray forms (sensitization phase) revealed the following: in groups of animals receiving the presumed therapeutic dose (3 µg/kg) of any of the test nasal spray forms, the paw mass ratio did not differ significantly from that of the control group. In contrast, animals receiving the highest tested dose (0.5 mg/kg) of the nasal spray forms showed a statistically significant increase in this parameter ($p < 0.05$) compared to both the control group and to animals receiving the same nasal spray form at the lower dose (Table 5).

Table 5. Reaction index based on paw mass ratio in the delayed-type hypersensitivity response after 10-day administration of the test forms during the sensitization phase; $n = 10$.

Group	Control Paw, g	Experimental Paw, g	Reaction Index Based on Paw Mass Ratio, %
Intact group	0.123 ± 0.0031	0.159 ± 0.0039	29.12 ± 4.26
Control group	0.129 ± 0.0032	0.161 ± 0.0033	27.69 ± 4.21
With copolymer (3 µg/kg)	0.121 ± 0.00294	0.151 ± 0.0030	21.78 ± 3.96
Without copolymer (3 µg/kg)	0.125 ± 0.0030	0.150 ± 0.0031	21.34 ± 2.64
With copolymer (0.5 mg/kg)	0.123 ± 0.0031	0.166 ± 0.0029	36.79 ± 4.09 *^
Without copolymer (0.5 mg/kg)	0.1231 ± 0.0030	0.165 ± 0.0029	36.04 ± 4.02 *^

Note: *— $p < 0.05$ versus the control group; ^— $p < 0.05$ versus animals receiving the same nasal spray form at a lower dose.

In the groups of animals receiving the nasal spray form at both the presumed therapeutic dose (3 µg/kg) and the highest tested dose (0.5 mg/kg), paw fluid content was higher compared to the control group, although the differences did not reach statistical significance. No differences were observed between the nasal spray forms at the same dose (Table 6).

Table 6. Reaction index based on paw fluid content in the delayed-type hypersensitivity response after 10-day administration of the test forms during the sensitization phase; n = 10.

Group	Fluid Content in the Control Paw, %	Fluid Content in the Experimental Paw, %	Reaction Index Based on Paw Fluid Content, %
Intact group	48.58 ± 0.30	50.42 ± 0.47	3.79 ± 0.62
Control group	49.41 ± 0.48	52.36 ± 0.48	5.96 ± 0.77
With copolymer (3 µg/kg)	48.34 ± 0.32	52.32 ± 0.56	8.29 ± 1.52
Without copolymer (3 µg/kg)	47.54 ± 0.24	51.46 ± 0.46	8.26 ± 0.95
With copolymer (0.5 mg/kg)	44.76 ± 0.76	48.24 ± 0.77	7.81 ± 0.85
Without copolymer (0.5 mg/kg)	44.23 ± 0.46	47.58 ± 0.55	7.58 ± 0.88

Analysis of the data obtained one day after the last administration of the nasal spray forms (provocation phase) showed that in the groups receiving either the presumed therapeutic dose (3 µg/kg) or the highest tested dose (0.5 mg/kg), the paw mass ratio was higher compared to the control group, although the differences did not reach statistical significance. No differences were observed between the nasal spray forms at the same dose (Table 7).

Table 7. Reaction index based on paw mass ratio in the delayed-type hypersensitivity response after a single administration of the test forms during the provocation phase; n = 10.

Group	Control Paw, g	Experimental Paw, g	Reaction Index Based on Paw mass Ratio, %
Intact group	0.14 ± 0.0020	0.17 ± 0.0044	21.39 ± 2.15
Control group	0.13 ± 0.0017	0.15 ± 0.0039	22.53 ± 2.94
With copolymer (3 µg/kg)	0.12 ± 0.0022	0.15 ± 0.0039	23.66 ± 2.08
Without copolymer (3 µg/kg)	0.13 ± 0.0031	0.16 ± 0.0049	26.12 ± 2.73
With copolymer (0.5 mg/kg)	0.12 ± 0.0022	0.15 ± 0.0032	22.60 ± 1.85
Without copolymer (0.5 mg/kg)	0.12 ± 0.0023	0.15 ± 0.0033	22.87 ± 1.81

In the groups of animals receiving any of the nasal spray forms at either the presumed therapeutic dose (3 µg/kg) or the highest tested dose (0.5 mg/kg), the paw fluid content ratio was significantly higher ($p < 0.05$) compared to the control group (Table 8). No statistically significant differences in paw swelling were observed between groups receiving different nasal spray forms at the same dose (Table 8).

Table 8. Reaction index based on paw fluid content in the delayed-type hypersensitivity response after a single administration of the test forms during the provocation phase; n = 10.

Group	Fluid Content in the Control Paw, %	Fluid Content in the Experimental Paw, %	Reaction Index Based on Paw Fluid Content, %
Intact group	55.05 ± 0.32	57.21 ± 0.37	3.92 ± 0.38
Control group	48.95 ± 0.48	51.57 ± 0.64	5.36 ± 0.69
With copolymer (3 µg/kg)	49.85 ± 0.27	55.06 ± 0.56	10.5 ± 1.08 *
Without copolymer (3 µg/kg)	49.34 ± 0.51	55.84 ± 0.76	13.2 ± 1.41 *
With copolymer (0.5 mg/kg)	48.37 ± 0.41	53.12 ± 0.58	9.8 ± 0.95 *
Without copolymer (0.5 mg/kg)	49.40 ± 0.38	53.51 ± 0.55	11.3 ± 0.92 *

Note: *— $p < 0.05$ versus the control group.

Thus, after 10 days of administration (sensitization phase), animals receiving any of the nasal spray forms at the highest tested dose (0.5 mg/kg) showed a significantly higher paw mass ratio ($p < 0.05$) compared to both the control group and the group receiving the

same nasal spray form at the therapeutic dose (3 µg/kg). After a single administration (provocation phase), animals receiving the nasal spray at either the therapeutic dose (3 µg/kg) or the highest tested dose (0.5 mg/kg) also exhibited a significantly higher paw fluid content ratio ($p < 0.05$) compared to the control group. No differences were observed between the nasal spray forms at the same dose.

The more pronounced effect of 10-day administration, reflected in changes in paw mass ratio, and the effect of single-day administration, assessed by the paw fluid content ratio, may be explained by differences in immune mechanisms at various stages of the inflammatory process. Administration of the nasal spray forms during the sensitization phase likely led to an increase in the number of immunoactive cellular elements, resulting in their more pronounced accumulation at the site of inflammation and, consequently, primarily affecting tissue mass. In contrast, administration of the nasal spray forms during the provocation phase was likely associated with an increase in the fluid component of the reaction, as reflected by changes in the paw fluid content ratio.

3.3. Effects of the Nasal Spray Forms on the Number of Antibody-Forming Cells

Analysis of the data obtained after 10 days of administration of the nasal spray forms (sensitization phase) revealed the following. In the spleens of intact animals, AFCs against sheep erythrocytes were not detected. In the control group, a moderate number of AFCs was observed. In the groups receiving the nasal spray forms for 10 days, a statistically significant increase in the number of AFCs in the spleen was observed compared to the control group ($p < 0.05$). No significant differences were found between the nasal spray forms at the same dose, either therapeutic (3 µg/kg) or highest tested (0.5 mg/kg) ($p > 0.05$). A statistically significant ($p < 0.05$) dose-dependent effect was also noted: animals receiving a higher dose of the same nasal spray form had a greater number of AFCs in the spleen (Table 9).

Table 9. Effect of both forms of the nasal spray on the humoral immune response following administration during the sensitization phase (10-day course) and the provocation phase (single dose); $n = 20$.

Group	Number of AFC During the Sensitization Phase (3 Days After Immunization)	Number of AFC During the Provocation Phase (5 Days After Immunization)
Intact group	Not detected	Not detected
Control group	4.55 ± 0.37	3.70 ± 0.37
With copolymer (3 µg/kg)	$9.85 \pm 0.62^*$	3.45 ± 0.34
Without copolymer (3 µg/kg)	$10.45 \pm 0.62^*$	3.20 ± 0.35
With copolymer (0.5 mg/kg)	$25.35 \pm 0.97^{*\wedge}$	3.55 ± 0.41
Without copolymer (0.5 mg/kg)	$27.10 \pm 0.83^{*\wedge}$	3.40 ± 0.28

Note: *— $p < 0.05$ versus the control group; ^— $p < 0.05$ versus animals receiving the same nasal spray form at a lower dose.

Analysis of the data obtained after a single administration of the nasal spray forms (provocation phase) showed that AFCs against sheep erythrocytes were not detected in the spleens of intact animals. In the control group, a moderate number of AFCs was observed. Animals receiving a single dose of the nasal spray, at both the therapeutic (3 µg/kg) and highest tested dose (0.5 mg/kg), also exhibited a moderate number of AFCs, although the differences compared to the control group did not reach statistical significance (Table 9).

Analysis of the number of AFCs per gram of spleen revealed a similar pattern. Administration of the nasal spray forms during the provocation phase had no substantial effect, whereas administration during the sensitization phase produced a pronounced dose-dependent effect (Table 10).

Table 10. Effect of both forms of the investigated nasal spray on the number of antibody-forming cells per gram of spleen following administration during the sensitization phase (10-day course) and the provocation phase (single dose); $n = 20$.

Group	Number of AFCs During the Sensitization Phase (3 Days After Immunization)	Number of AFCs During the Provocation Phase (5 Days After Immunization)
Intact group	Not detected	Not detected
Control group	367 ± 34	291.65 ± 28.33
With copolymer (3 $\mu\text{g/kg}$)	$764 \pm 49^*$	266.8 ± 26.59
Without copolymer (3 $\mu\text{g/kg}$)	$802 \pm 48^*$	263.25 ± 29.16
With copolymer (0.5 mg/kg)	$2028 \pm 99^{*\wedge}$	275.44 ± 33.57
Without copolymer (0.5 mg/kg)	$2099 \pm 84^{*\wedge}$	285 ± 24.25

Note: $^* - p < 0.05$ versus the control group; $^\wedge - p < 0.05$ versus animals receiving the same nasal spray form at a lower dose.

3.4. Efficacy of the Nasal Spray and Its Combination with the Copolymer After Intranasal Administration in a SARS-CoV-2 Infection Model

Clinical signs of infection in the viral control group began to appear on day 3 of the experiment. Animals in this group exhibited hyperemia of the nasal planum and nasal mucosa, as well as increased secretion from the nasal glands. Their activity was decreased, food and water intake were reduced, and responses to external stimuli were delayed. In contrast, in the groups receiving the nasal spray forms under different dosing regimens (groups 1–4), no clinical signs of infection were observed on day 3 post-infection. The animals remained in a stable condition, consistent with physiological norms, responded promptly to stimuli, had clear airways without drops of mucous exudate, and the nasal mucosa appeared moderately moist and light pink in color.

By the end of the experiment, on day 6 post-infection, clinical signs of viral respiratory infection were observed in all animals. In the viral control group (group 5), frequent and prolonged sneezing with abundant exudate and hyperemia of the nasal planum were noted, along with occasional coughing. Activity in this group was reduced; animals responded sluggishly to stimuli, and food intake was decreased. In contrast, in groups 1–4, clinical signs of infection were milder than in the viral control group, and no coughing was observed in any animal. The most pronounced effect was seen with the nasal spray form containing the copolymer administered according to the therapeutic–prophylactic regimen (group 4).

Daily monitoring of body weight revealed differences depending on the dosing regimen and the presence of the copolymer. Animals in the viral control group (group 5) lost 2% of their body weight by day 4. In group 4, receiving the form with the copolymer under the therapeutic–prophylactic regimen, animals gained weight actively, similar to the non-infected intact group, and the percentage of weight gain was statistically higher than in the viral control group ($p < 0.05$). In the remaining experimental groups, body weight remained stable throughout the study, fluctuating within $\pm 2\%$ (Figure 2).

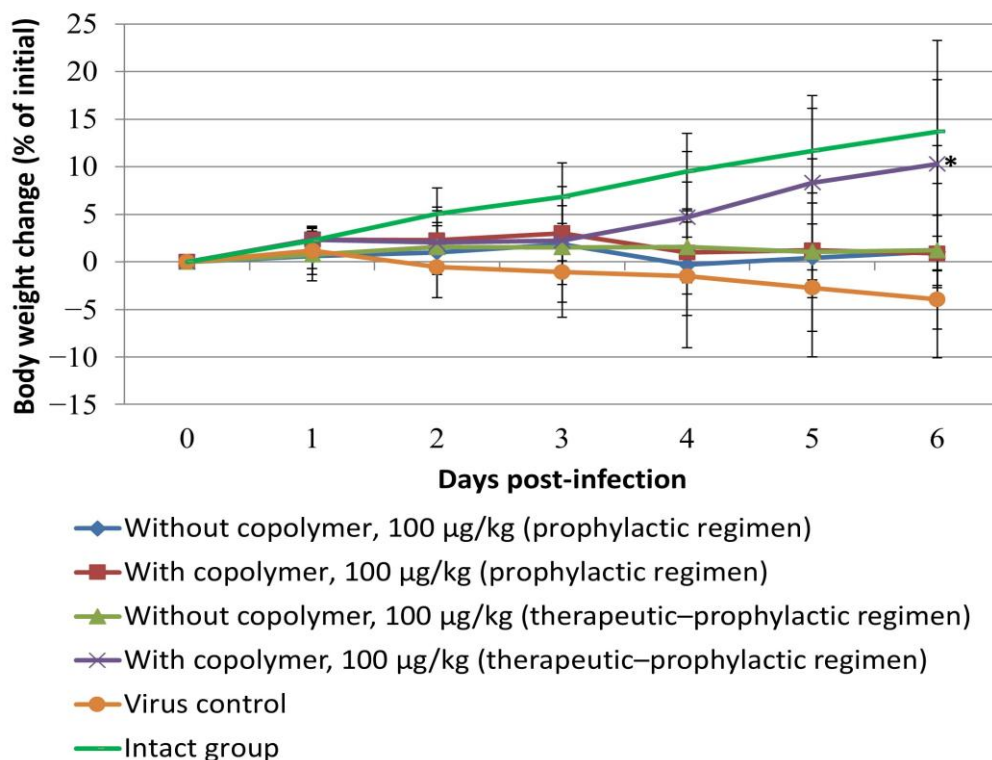


Figure 2. Body weight dynamics of Syrian hamsters in the SARS-CoV-2 infection model. Asterisks indicate significant differences compared with the viral control group, determined using the Kruskal–Wallis test followed by Dunn’s post hoc test with Bonferroni correction, * $p < 0.05$.

On day 3 post-infection, the WLI in the viral control group (group 5) was elevated, reaching 14 points. In contrast, administration of the nasal spray and its combination with the copolymer under both dosing regimens effectively protected the animals from pulmonary edema. WLI values were comparable to those in the non-infected intact group, and therefore did not suggest marked lung weight increase (Figure 3).

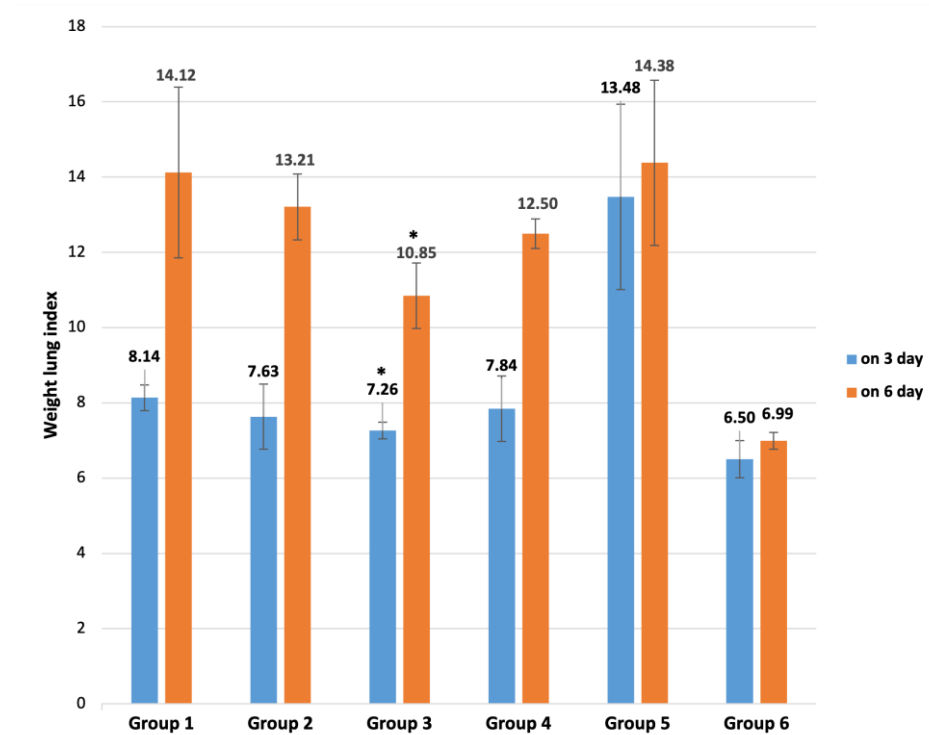


Figure 3. Weight lung index of Syrian hamsters in the SARS-CoV-2 infection model. Group 1, nasal spray without copolymer administered according to the prophylactic regimen; Group 2, nasal spray with copolymer administered according to the prophylactic regimen; Group 3, nasal spray without copolymer administered according to the therapeutic–prophylactic regimen; Group 4, nasal spray combined with copolymer administered according to the therapeutic–prophylactic regimen; Group 5, viral control. Group 6, intact group. Asterisks indicate significant differences compared with the viral control group, determined using the Kruskal–Wallis test followed by Dunn’s post hoc test with Bonferroni correction; * $p < 0.05$.

By day 6 post-infection, WLI increased, but in the groups receiving the nasal spray form or its combination with the copolymer (groups 1–4), it remained lower than in the viral control group. The effect was most evident in groups 3 and 4, while a statistically significant reduction compared with the viral control group was observed in group 3 ($p < 0.05$) (Figure 3).

The decrease in WLI in animals receiving the nasal spray forms may reflect reduced interalveolar tissue edema, lower exudate accumulation in the bronchi and alveoli, and slower progression of inflammation in lung tissue compared with the viral control group.

On day 3 post-infection, the viral titer in the viral control group (group 5) averaged 7.52 ± 0.2 log TCID₅₀/0.1 mL. Administration of the nasal spray forms reduced viral load in the lungs (Figure 4).

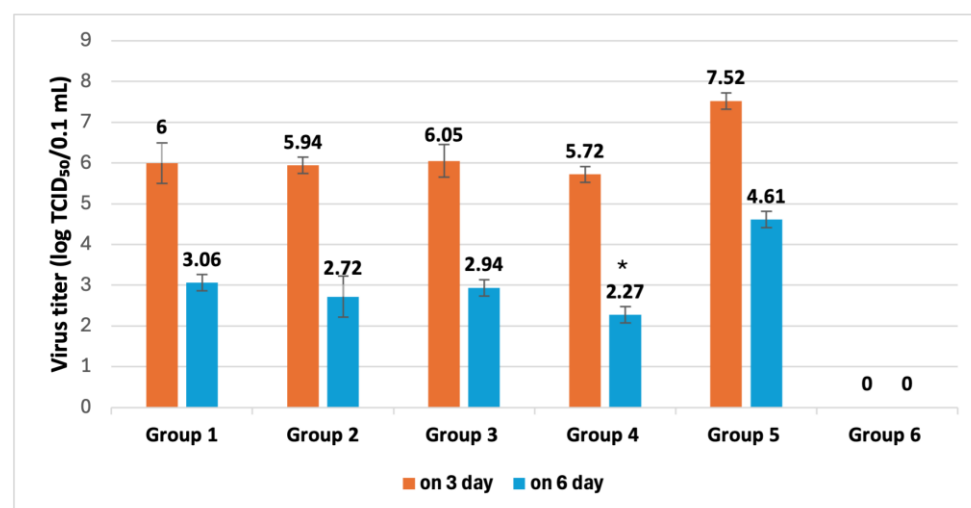


Figure 4. Virus titer in hamster lungs on days 3 and 6 after SARS-CoV-2 infection. Asterisks indicate significant differences compared with the viral control group, determined using the Kruskal–Wallis test followed by Dunn’s post hoc test with Bonferroni correction, * $p < 0.05$.

By day 6 post-infection, the viral titer in group 5 decreased to 4.61 ± 0.2 log TCID₅₀/0.1 mL. Analysis of lung tissues from the experimental groups showed that administration of the nasal spray and its combination with the copolymer under both dosing regimens effectively inhibited SARS-CoV-2 replication in the lungs, with reductions exceeding 1.5 log TCID₅₀/0.1 mL compared to the viral control group. A statistically significant reduction in viral titer compared with the viral control group was observed in group 4 ($p < 0.05$). As on day 3, the therapeutic–prophylactic regimen remained the most effective, resulting in the greatest reduction in lung viral load—by 2 log TCID₅₀/0.1 mL relative to the viral control group (Figure 4).

3.5. Histopathological Analysis of Lung Tissue

In the intact group (group 6), lung histology corresponded to that of a healthy animal. The architecture of the alveolar tissue was preserved, alveoli had clear contours, and no destructive changes were observed (Figure 5F). No inflammatory reaction was detected. Cell density within a standard area of the section was 48.8 ± 0.13 cells/ μm^2 , alveolar septal thickness was 4.09 ± 0.04 μm , and the height of the bronchial epithelium in a small bronchus was 16.15 ± 0.02 μm (Table 11).

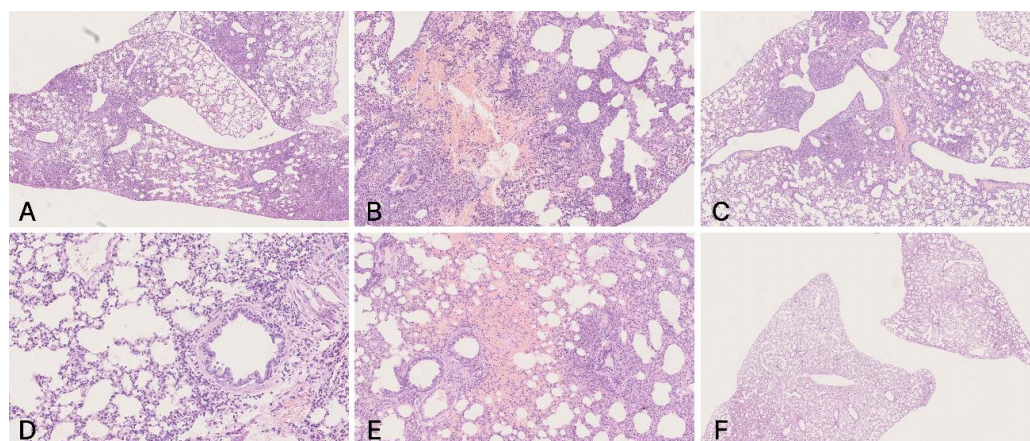


Figure 5. Morphological changes in hamsters' lungs at 6 days post-infection with SARS-CoV-2 and intranasal administration of the nasal spray forms, $n = 3$. (A) Group 1, nasal spray without copolymer administered according to the prophylactic regimen; (B) Group 2, nasal spray with copolymer administered according to the prophylactic regimen; (C) Group 3, nasal spray without copolymer administered according to the therapeutic–prophylactic regimen; (D) Group 4, nasal spray combined with copolymer administered according to the therapeutic–prophylactic regimen; (E) Group 5, viral control; (F) Group 6, intact group. Hematoxylin and eosin staining. Magnification $\times 100$ (F), $\times 200$ (A,C), $\times 300$ (B,D,E).

Table 11. Morphometric parameters of lung tissue in Syrian hamsters.

Group	Morphometric Parameters		
	Cell Density in the Inflammatory Infiltrate, μm^2	Thickness of Inter-alveolar Septa, μm	Height of the Epithelium in Small Bronchi, μm
Group 1. Without copolymer, 100 $\mu\text{g}/\text{kg}$ (prophylactic regimen)	78.27 ± 0.15	6.09 ± 0.04	12.83 ± 0.08
Group 2. With copolymer, 100 $\mu\text{g}/\text{kg}$ (prophylactic regimen)	71.33 ± 0.11	5.43 ± 0.03	12.65 ± 0.04
Group 3 Without copolymer, 100 $\mu\text{g}/\text{kg}$ (therapeutic–prophylactic regimen)	56.27 ± 0.08	4.89 ± 0.05	11.71 ± 0.04
Group 4 With copolymer, 100 $\mu\text{g}/\text{kg}$ (therapeutic–prophylactic regimen)	62.47 ± 0.09	4.78 ± 0.05	12.81 ± 0.02
Group 5 Virus control	75.27 ± 0.01	6.78 ± 0.03	12.35 ± 0.02
Group 6 Intact group	48.8 ± 0.13	4.09 ± 0.04	16.15 ± 0.02

Infection with SARS-CoV-2 (viral control, group 5) induced pronounced pathological changes in the lungs, including diffuse and focal inflammatory infiltrates, thickening of the alveolar septa, signs of endotheliitis, and focal hemorrhages (Figure 5E). This was reflected in an increased cell density within the inflammatory infiltrate to 75.27 ± 0.01 cells/ μm^2 , alveolar septal thickness to 6.78 ± 0.03 μm , and a decrease in bronchial epithelial height in small bronchi to 12.35 ± 0.02 μm compared with the intact group (Table 11).

Prophylactic intranasal administration of the investigated nasal spray forms for 5 days prior to infection (groups 1 and 2) did not result in a marked reduction in inflammatory changes. Histologically, extensive areas of cellular infiltration and signs of an active inflammatory response were observed, which may reflect the activation of innate immune mechanisms at the early stages of infection (Figure 5A,B).

In contrast, the therapeutic–prophylactic regimen of nasal spray administration (groups 3 and 4), which included dosing 3 days prior to infection, 1 h before infection, and for 3 consecutive days post-infection, was associated with a reduction in the intensity of inflammatory changes. In group 3, which received the nasal spray form without the copolymer, inflammation was predominantly confined to peribronchial areas, with a decrease in edema and reduced density of the cellular infiltrate (Figure 5C, Table 11).

The most pronounced protective effect was observed in group 4, which received the nasal spray form combined with the copolymer. In this group, the overall structure of the respiratory acini was largely preserved, inflammatory infiltrates were less extensive, and vascular alterations were less pronounced. Additionally, active proliferation of type II pneumocytes was noted, indicating activation of reparative processes in the lung tissue (Figure 5D). As a result, morphometric parameters of the inflammatory process in these groups approached those of the intact group, although they did not fully reach physiological levels (Table 11).

3.6. Efficacy of the Nasal Spray and Its Combination with a Copolymer upon Intranasal Administration in a Mouse Model of Influenza Infection

In the intact group (Group 6), all animals survived until the end of the experiment and exhibited steady body weight gain. In the viral control group (Group 5), 100% mortality was observed by day 8, with the MST of approximately 6 days (Figure 6), and body weight decreased by up to 29% at the time of death (Figure 7).

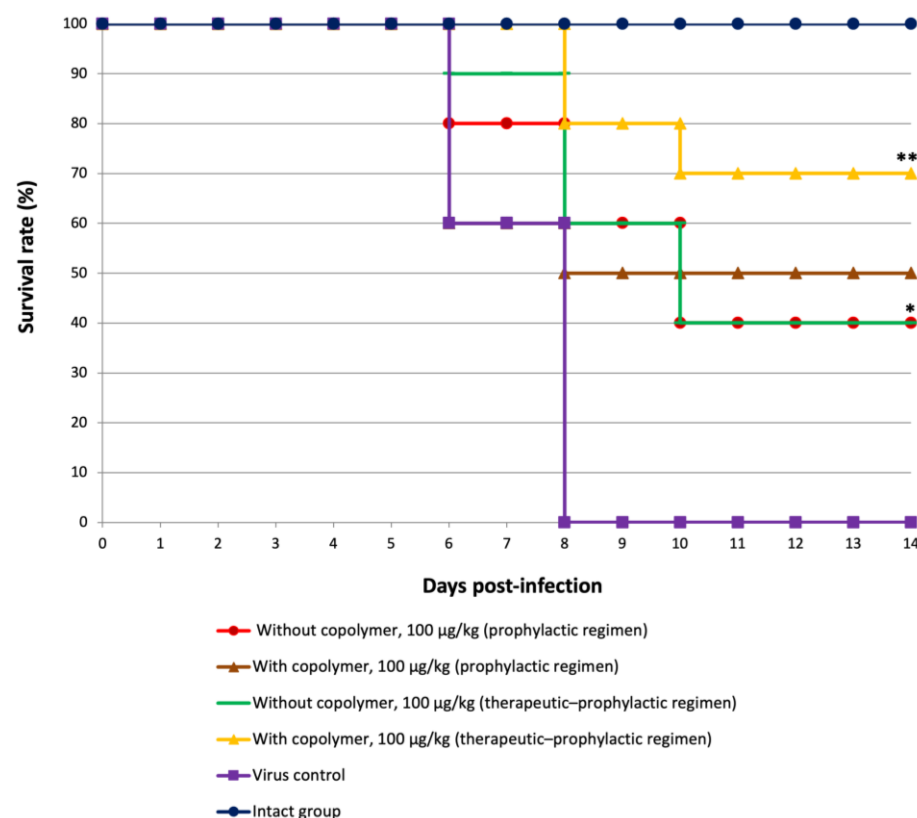


Figure 6. Survival rate of mice in the influenza A-induced pneumonia model, presented as stepwise Kaplan–Meier curves. Asterisks indicate significant differences compared with the viral control group, as determined by the log-rank test with Bonferroni adjustment, * $p < 0.05$; ** $p < 0.001$.

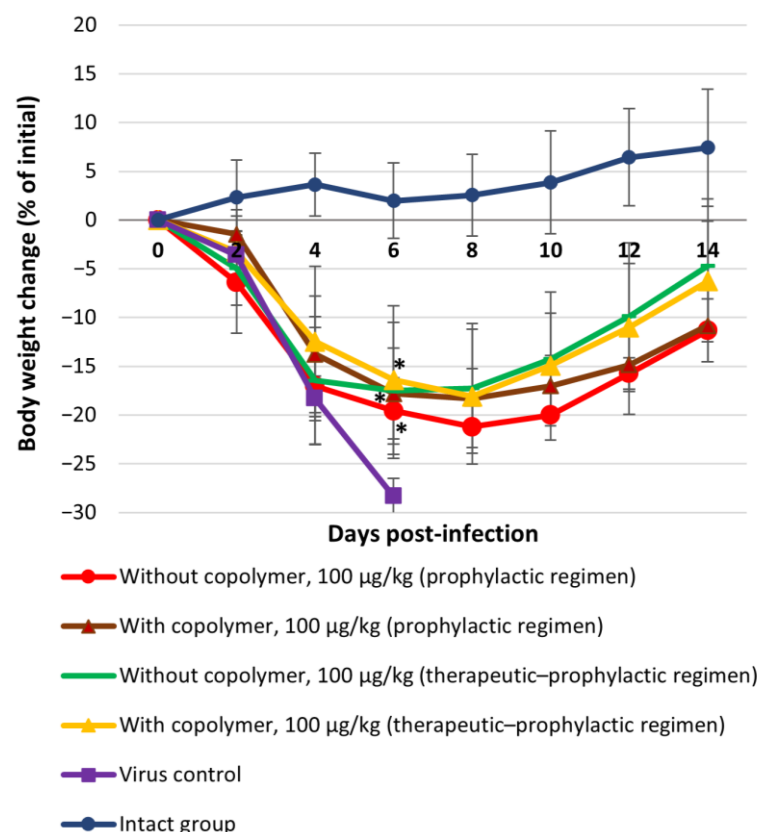


Figure 7. Body weight dynamics of mice in the influenza A-induced pneumonia model. Asterisks indicate significant differences compared with the viral control group, determined using the Kruskal–Wallis test followed by Dunn’s post hoc test with Bonferroni correction; * $p < 0.05$.

Administration of the nasal spray, both alone and in combination with the copolymer, was effective in the lethal influenza infection model induced by influenza virus A/California/04/2009, depending on the treatment regimen and the presence of the copolymer.

When the nasal spray form without copolymer was administered under both regimens, mouse survival increased to 40%. In Group 1, treated according to the prophylactic protocol, the MST reached 9.4 days, whereas in the therapeutic–prophylactic group (Group 3), the MST was slightly higher and reached 9.6 days (Figure 6). Kaplan–Meier analysis showed a significant improvement in survival in both groups compared with the viral control group ($p < 0.05$). Body weight loss on day 6 was reduced to approximately 20% in Group 1 and 17.49% in Group 3; the reduction was statistically significant for Group 1 compared with the viral control group ($p < 0.05$) (Figure 7).

The addition of the copolymer further enhanced efficacy. In Group 2, 50% of the animals survived, the MST was 9.2 days (Figure 6), and body weight loss on day 6 was reduced to 18%, which was significantly lower than in the viral control group ($p < 0.05$) (Figure 7).

The most pronounced protective effect was observed in animals receiving the nasal spray combined with the copolymer under the therapeutic–prophylactic regimen (Group 4). In this group, survival reached 70%, and the MST increased to 11.4 days compared with 6 days in the viral control group (Figure 6). Kaplan–Meier analysis showed a significant improvement in survival compared with the viral control group ($p < 0.001$). Body weight loss decreased to 16.42%, which was also significantly lower than in the viral control group ($p < 0.05$) (Figure 7).

The level of viral replication in the lungs corresponded to the data on survival, body weight loss, and MST. In the viral control group, where all animals succumbed, the viral titer reached a maximum of $\geq 7 \pm 0 \log_{10}$ TCID₅₀/0.1 mL. Administration of the nasal spray forms affected viral replication in the lungs depending on the dosing regimen and the presence of the copolymer. The most pronounced suppression of viral replication was observed with the copolymer-containing nasal spray form under the therapeutic–prophylactic regimen (Group 4), with viral titers reduced to $3.9 \pm 0.9 \log_{10}$ TCID₅₀/0.1 mL. This reduction was statistically significant compared with the viral control group ($p < 0.001$) (Figure 8).

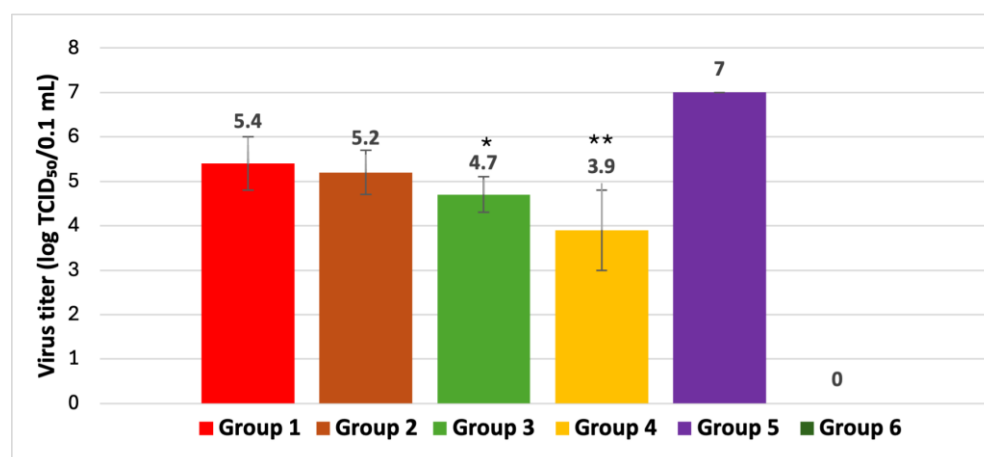


Figure 8. Virus titer in mouse lungs on day 4 after influenza A infection. Group 1, nasal spray without copolymer administered according to the prophylactic regimen; Group 2, nasal spray with copolymer administered according to the prophylactic regimen; Group 3, nasal spray without copolymer administered according to the therapeutic–prophylactic regimen; Group 4, nasal spray combined with copolymer administered according to the therapeutic–prophylactic regimen; Group 5, viral control. Group 6, intact group. Asterisks indicate significant differences compared with the viral control group, determined using the Kruskal–Wallis test followed by Dunn’s post hoc test with Bonferroni correction, * $p < 0.01$, ** $p < 0.001$.

4. Discussion

Both forms of the investigated nasal spray, with and without the copolymer, demonstrated comparable immunostimulatory properties. The nasal spray forms induced a dose-dependent enhancement of both cellular and humoral immune responses, manifested by an increased number of antibody-forming cells and activation of inflammatory reactions at different stages of the immune process (sensitization or provocation). The obtained results are consistent with literature data indicating the ability of bacterial lysates to stimulate various components of the immune response [32–36].

The results of phagocytic activity assessment may indicate an effect of the nasal spray forms on the cellular component of the immune response. A preliminary 10-day course of nasal spray administration probably provided a more controlled leukocyte response to subsequent peptone exposure, which was reflected by lower phagocytic activity compared with the control group, while maintaining a dose-dependent pattern. These findings suggest that the nasal spray forms may influence regulation of the inflammatory response. However, further studies are required to clarify the underlying mechanisms.

Based on the combined assessment of viral titers, body weight dynamics, survival, WLI, and lung pathology, the nasal spray forms demonstrated an antiviral effect in the experimental infection models. This effect may be associated with modulation of innate immune responses, which can contribute to restriction of viral replication and support subsequent adaptive immune responses. These findings are consistent with previously

reported effects of bacterial lysates used for the prevention and treatment of respiratory infections, which may enhance nonspecific defense mechanisms and contribute to protection against respiratory pathogens primarily through innate immune activation [19–21,37,38]. However, further mechanistic studies, including analysis of cytokine profiles, mucosal immune responses, and cellular activation markers, are required to clarify the pathways underlying the observed antiviral effect.

The use of the copolymer of 2-methyl-5-vinylpyridine and N-vinylpyrrolidone hydrochloride appeared to enhance and stabilize the local immune response by prolonging contact between antigenic components and the nasal epithelium. This effect is likely associated with the induction of mechanisms resembling the phenomenon of trained immunity, a state in which innate immune cells acquire enhanced functional readiness upon repeated pathogen exposure [39,40].

At the same time, the absence of pronounced differences between the nasal spray forms with and without the copolymer suggests that the antigenic component is the key determinant of the immunomodulatory effect, driving activation of innate immunity and subsequent engagement of the adaptive immune response. This is supported by the observed reduction in viral replication in the lungs and the magnitude of the prophylactic effect, which is particularly important for preventing the development of respiratory infections.

Several limitations of this study should be acknowledged. First, although the resource-equation approach supported the overall animal-use design, the number of animals at each terminal time point in the SARS-CoV-2 model was limited ($n = 3$ per group), which limits the statistical power of pairwise comparisons. Therefore, statistically significant differences observed for individual endpoints should be interpreted with caution and considered together with the overall consistency of clinical, virological, and pathological findings. Second, the infection models did not include a standard-of-care antiviral comparator as a positive control. Accordingly, the magnitude of the protective effect relative to approved antiviral treatment could not be directly assessed. Thus, the study provides proof-of-concept evidence and supports further confirmatory studies with larger cohorts and optimized comparator designs.

5. Conclusions

The present findings support further investigation of the nasal spray forms as potential candidates for prophylaxis and adjunctive therapy of respiratory infections, particularly in settings where pathogen variability may limit the effectiveness of pathogen-directed treatments.

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Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AFC	Antibody-forming cells
DTH	Delayed-type hypersensitivity responses
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
MST	Mean survival time
PBS	Phosphate-buffered saline
TCID	Tissue Culture Infectious Dose
WLI	Weight lung index

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