Virology Journal

RESEARCH

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Evaluation of the duration of new nasal drug interferon α-2b activity in an experimental model system

N. Bezdieniezhnykh[1*], O. Lykhova[1], G. Borshchevskiy[3], Ye. Kruglov[2] and M. Borshchevska[3]

Abstract

This study aimed To develop an adequate system for assessing the duration of IFN antiviral activity in the nasal cavity of animals and to study the antiviral activity of a new IFN drug in the form of a gel in comparison with the known spray form.

Methods To assess the duration of the specific activity of IFN, the drugs were administered into the nasal cavity of experimental rats. One, 3, and 4 h after administration, the nasal cavities of the rats were washed out, and the antiviral activity of washed from the nasal cavity (WNC) was analyzed in vitro.

Results Developed an algorithm to assess the duration of antiviral activity of nasal preparations using a sensitive in vitro test system, in which the analysis of the antiviral activity of WNC made it possible to detect antiviral activity after the administration of nasal forms of IFN—a new variant in the form of a gel in comparison with the IFN spray. It was established that 1 h after the intranasal administration of IFN, the same antiviral activity and IFN concentration is: 266.8 ± 14.0 IU/ml for IFN/spray and 260.2 ± 20.9 IU/ ml for IFN/gel. Three hours after the drugs are administered, their activity decreases but remains at a sufficiently high level: 121.4 ± 5.4 IU/ml for IFN/spray and 88.3 ± 6.2 IU/ml for IFN/gel. Four hours after IFN administration, the concentration of IFN/gel was significantly greater than that of IFN/spray: 39.4 ± 4.9 IU/ml and 10.6 ± 1.0 IU/ml, respectively.

Conclusion The developed system for evaluating the antiviral activity of nasal preparations allows the study of the duration of the local antiviral effect of drugs. The antiviral activity of the IFN nasal preparations persisted for 4 h after their intranasal administration: the concentration of IFN in gel form on the walls of the nasal cavity was significantly greater than that of the IFN spray.

Keywords Interferon, Antiviral activity, Barrier, Nasal spray, Gel

Introduction

For the first time, interferon was described as an antiviral agent by Isaacs and Lindenmann in 1957. IFN is a ubiquitous cytokine that controls a cascade of events at the molecular-genetic, cellular, and systemic levels, preventing cell transformation [1]. By nature, IFNs are tissue hormones characterized by their polypotent action: they can induce resistance to a wide range of viruses, inhibit cell proliferation, and modify the surface properties of normal and tumor cells [2]. Owing to the well-known antiviral effect of IFN, it is currently a potential

*Correspondence:
N. Bezdieniezhnykh
beznaliano@gmail.com; beznaliano@ukr.net
[1] R.E.Kavetsky Institute of Experimental Pathology, Oncology, and Radiobiology of the NAS of Ukraine, Kyiv, Ukraine
[2] National University of Pharmacy of the Ministry of Health of Ukraine, Kyiv, Ukraine
[3] JSC Farmak, Kyiv, Ukraine

prophylactic agent during pandemics, including the example of the latest pandemic of coronavirus disease 2019 [3–6], and the simplest solution for the use of this cytokine is its nasal form since this is a variant of the use of IFN that acts locally, is characterized by ease of introduction and creates a primary "antivirus" barrier. However, the time at which the drug is in the nasal cavity is extremely important, which is why the search for ways to modify nasal forms of IFN to increase the period of local action and its barrier function with antiviral properties is relevant today [7].

In addition, there is a problem of the correct assessment of the antiviral effect duration of intranasal drugs, which can adequately and informatively register the presence of the corresponding drug in the nasal cavity for the formation of the most effective drug application scheme. That is why it became one of the main tasks of the work: to develop an effective and correct algorithm for studying the duration of action of nasal drugs with the possibility of assessing the antiviral activity of drugs dynamically.

The development of the composition of Interferon α−2b, in the form of a nasal spray, ensures long-term retention of the active pharmaceutical ingredient (API) on the mucous membrane, was implemented by JSC Farmak with the creation of an innovative composition of the medicinal product based on a colloidal solution – a sol that turns into a gel after spraying in the nasal cavity. The composition of the drug contains a combination of high-molecular compounds that form a gel with thixotropic properties, which, under the influence of mechanical forces (shear forces), for example, as a result of shaking, pressure through a nozzle or mixing, turns into a liquid sol and forms a gel again in a state of rest. The obtained sample of the developed drug Interferon α−2b 100,000 IU/ml, nasal spray (in the form of a gel) (JSC Farmak) was evaluated for the duration of activity in the nasal cavity in comparison with the drug Nazoferon 100,000 IU/ml, nasal spray (JSC Farmak).

Materials and methods

Wistar rats (females), weighing 170–200 g, and aged 2.5–3.0 months, were used. The research was conducted in compliance with the basic requirements for the maintenance and handling of laboratory animals and the provisions of the European Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes (Strasbourg, 1986). All procedures involving experimental animals were reviewed and approved by the Commission on Bioethics of R.E.Kavetsky Institute of Experimental Pathology, Oncology, and Radiobiology (IEPOR), the NAS of Ukraine in compliance with the ethical principles of working with animals. All animal experiments were performed according to the ARRIVE guidelines and recommendations (study design, sample size, inclusion and exclusion criteria, randomization, blinded analysis, outcome assessment, and statistical methods).

Cell culture and virus: immortalized bull kidney cells (MDBK line), and vesicular stomatitis virus (VSV) (Indiana strain). The cell line and virus strain were obtained from the Bank of Cell Lines from Human and Animal Tissues of the R.E. Kavetsky IEPOR (Cabinet of Ministers of Ukraine Resolution No. 1709 of December 19, 2001, Certificate of Registration of the National Academy of Sciences, Series No. 41 of February 19, 2009).

Reagents: High-glucose DMEM (Biowest, France, cat. no. L0102-500), newborn calf serum (NCS) (Biowest, France, cat. no. S0750-500), Versen's solution (Vetline Agroscience, Ukraine, series 1), physiological solution (Lekhim, Ukraine, series 71,033,007), gentamicin (Sigma, USA, cat. no. G1397), crystal violet (Sigma, USA, cat. no. C6158), and trypan blue (Applichem, Germany, cat. no. A0668) were used.

To determine the antiviral activity of the studied samples in a sensitive cell system in vitro, the 2nd WHO International Standard 1999 was used as the interferon-α standard. Interferon α 2b Human, rDNA *E. coli* derived 95/566, 70,000 IU per ampoule.

Study drugs: human recombinant interferon-α 2b (IFN) medicinal products, nasal form:

No. 1. Interferon-α 2b nasal spray (Nazoferon) (JSC Farmak) – hereinafter referred to as "Spray", initial concentration of IFN 100,000 IU/ml.

No. 2. Interferon-α 2b nasal spray (in the form of a gel) (JSC Farmak) – hereinafter referred to as "Gel", initial concentration of IFN 100,000 IU/ml.

Intranasal administration of Interferon α−2b "Spray" and "Gel" and washing from the nasal cavity (WNC) of experimental animals after drug administration

To determine the duration of the specific (antiviral) activity of the IFN nasal preparations "Spray" and "Gel" in the in vivo system, the drugs were administered to experimental animals intranasally (into the nasal cavity) in a total volume of 100 µl of the original drug: 50 µl in each nostril. The drug was administered once, after which several procedures of WNC in the experimental animals were carried out via an automatic pipette at scheduled time intervals (1, 3 and 4 h). The nasal cavity of each animal was washed with sterile physiological solution in a volume of 300 µl/animal. Thirty-nine Wistar rats (females) were used in the study. The study was conducted with the participation of 9 groups of animals: 6 experimental and 3 control groups. The scheme of the experiment (groups of animals and their numbers) is given below (Table 1).

Table 1 Expermimental design

Group, №	Test drug "Spray" or "Gel"	Time after administration of nasal IFN into the nasal cavity of the rat / WNC	Number of animals
1.1	"Spray"	1 h	5
1.2	"Spray"	3 h	5
1.3	"Spray"	4 h	5
2.1	"Gel"	1 h	5
2.2	"Gel"	3 h	5
2.3	"Gel"	4 h	5
3.1	Control*	1 h	3
3.2	Control*	3 h	3
3.3	Control*	4 h	3

*- *physiological solution*

Test spray and gel are stored in a place protected from light at a temperature of 2 to 8°C. After opening the bottle, the medicines retain their stability and activity for 10 days when stored in a refrigerator (at 2—8 °C). Warmed the drug to room temperature before administration.

Evaluation of the in vitro *antiviral effect of rats' WNC after the administration of nasal IFN*

Cultivation of cells. MDBK cells were cultured in complete nutrient medium (DMEM) supplemented with 10% NCS and 40 μg/ml gentamicin in plastic dishes for cell culture in a humidified atmosphere at 5% CO_2 and 37 °C. The medium was changed, and the cells were reseeded according to the standard method [8]. The cells were subcultured after they reached 80–90% confluence of the monolayer via Versen's solution. The experiments used cells that were in the exponential phase of growth.

Virus. Vesicular stomatitis virus Indiana strain is a virus in the family Rhabdoviridae.

The virus infects cattle and can infect humans, causing illness with flu-like symptoms or encephalitis. Vesiculoviruses are relatively simple, having a linear, single stranded, negative sense RNA genome encased in a bullet-shaped virion made from only five proteins. Upon infection of cultured cells, viral products turn off cellular gene expression and seize the entire metabolic potential of the cell. They also depolymerize the cytoskeleton to cause rapid tissue destruction [9]. The VSV was developed on Vero cells according to the standard method [10] and stored in liquid nitrogen. The titer of VSV on the MDBK cells was 5×10^8 PFU/ml.

Evaluation of the antiviral activity of WNC via *a sensitive cellular test system.* The concentration of IFN-α in the test samples (WNC) was determined by its antiviral activity with a standard micromethod using MDBK cells and VSV as a test system. To determine the antiviral activity of IFN MDBK cells were seeded on a 96-well plate at a concentration of 2×10^4 cells/well in a complete DMEM with 5% NCS and 40 μg/ml gentamicin and incubated at 37 °C and 5% CO_2. After 24 h, the test samples and human IFN-α standard were introduced into the corresponding wells and the cells were incubated at 37 °C in the presence of 5% CO_2 for another 24 h. After one day, VSV was added to all wells, except for the control cells, at a concentration of 100 cytopathic effect) in DMEM with 2% NCS, and the cells were incubated at 37 °C and 5% CO_2. After 24 h, the obtained results were registered visually (direct microscopy method) and colorimetrically by staining the cells with crystal violet. The unit of activity was taken as the inverse of the last dilution of the drug, which protected 50% of the cells from the cytopathic effect of VSV. The concentration of IFN-α was expressed in international units per ml of fluid (IU/ml).

Colorimetric method for evaluating the results. After the end of incubation live cells were stained with crystal violet. The nutrient medium was removed from the plates and 50 μl of crystal violet solution (5 mg dye in 1 ml of 70% methyl alcohol) was added to each well. After 10 min, the dye was washed three times with running water. After that, the plate was dried at room temperature and the dye was eluted with 96% ethyl alcohol (100 μl/well) for 10 min using a PSU-2 T mini shaker at 300 rpm. The results were recorded via a spectrophotometer with a vertical beam path at a wavelength of 540 nm. The number of living cells (X) in each well of the plate was calculated as a percentage, via the following formula: $X = \frac{A_1 \cdot 100\%}{A_0}$, where A0 is the average value of the optical density in the wells of the negative control, and A1 is the value of the optical density in the well of the experimental group.

Statistical analysis. Calculations of the average value of the studied indicators (M) and the average error of the arithmetic mean (m) were performed via the Excel 2016 software package. The nonparametric Mann–Whitney U test was used to assess the level of statistical significance of the differences in the studied indicators between groups. Calculations were performed via the STATISTICA 6.0 software package. Differences between groups were considered statistically significant if the obtained probability coefficient p was less than the significance level α 0.05.

Results

The results of antiviral activity were obtained in the WNC of rats after intranasal administration of Interferon α−2b "Spray" or Interferon α−2b "Gel" (Fig. 1).

It was established that 1 h after the intranasal administration of IFN drugs (10,000 IU/animal), the same antiviral activity was observed in the WNC of the rats in both experimental groups and the concentration of IFN was 266.8 ± 14.0 IU/ml for the IFN/spray group and 260.2 ± 20.9 IU/ml for the IFN/gel group (Fig. 2A, 3). Three hours after the intranasal administration of the studied drugs, although the antiviral activity of the WNC of the experimental groups decreased, it remained high: 121.4 ± 5.4 IU/ml for the IFN/spray group and 88.3 ± 6.2 IU/ml for the IFN/gel group (Fig. 2B, 3). The results revealed that the concentration of IFN was significantly lower in the IFN/gel group than in the IFN/spray group, which, in our opinion, is related to the specific structure and consistency of the drug in the form of a gel, which has a viscous structure, as it is a gel-like substance that contributes to closer contact with the mucous membrane of the nose and, accordingly, to the complication of rinsing. This structure contributes to a longer preservation of the drug in the site of contact and, potentially, a longer specific (local) activity, which is confirmed by the assessment of IFN activity in the WNC after 4 h: a statistically significantly higher concentration of IFN (in IU/ml) was observed in the WNC of the rats with IFN/gel - 39.4 ± 4.9 IU/ml relative to those in IFN/spray group - 10.6 ± 1.0 IU/ml (Fig. 2C, 3). Additionaly the results suggest that the antiviral activity of IFN preparations is preserved in the nasal cavity of experimental animals for at least 4 h after their intranasal administration. Moreover, the assessment of IFN in the control group (intranasal

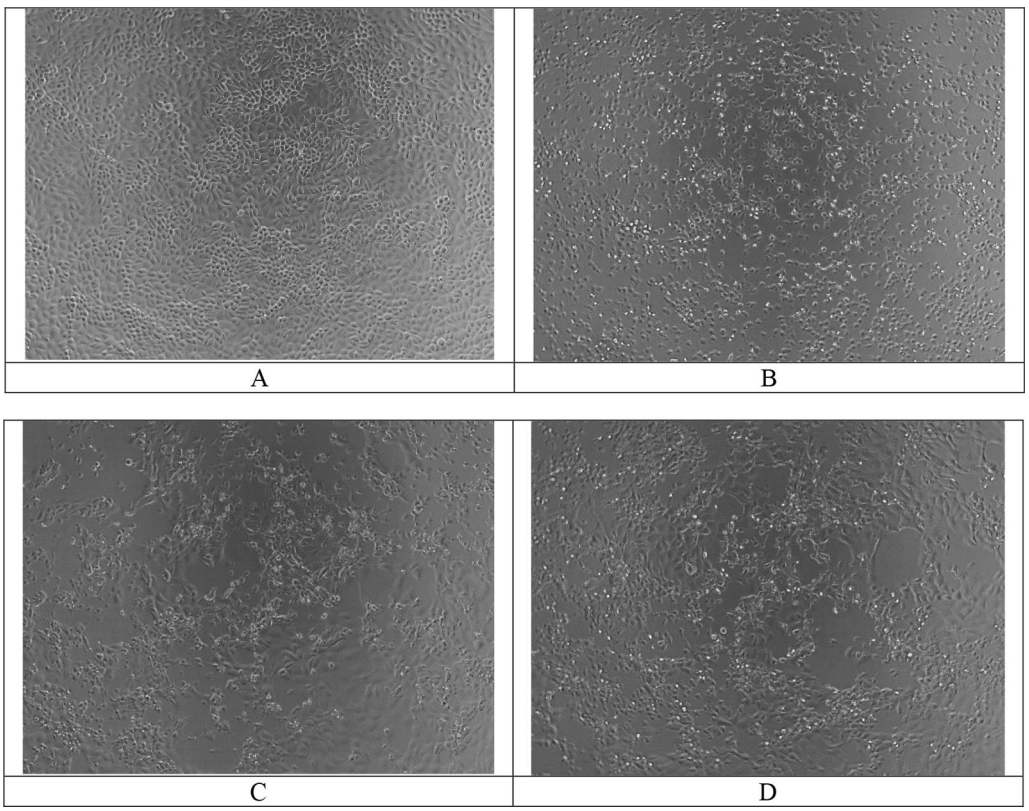

Fig. 1 Antiviral activity of IFN-alpha in a sensitive cell test system in vitro: **A** MDBK cells. Negative control; **B** MDBK + VSV, cytopathic effect. Positive control (VSV control); **C** MDBK cells + Sprey + VSV (50% damage to the cell monolayer by VSV); **D** MDBK cells + Gel + VSV (50% damage to the cell monolayer by VSV)

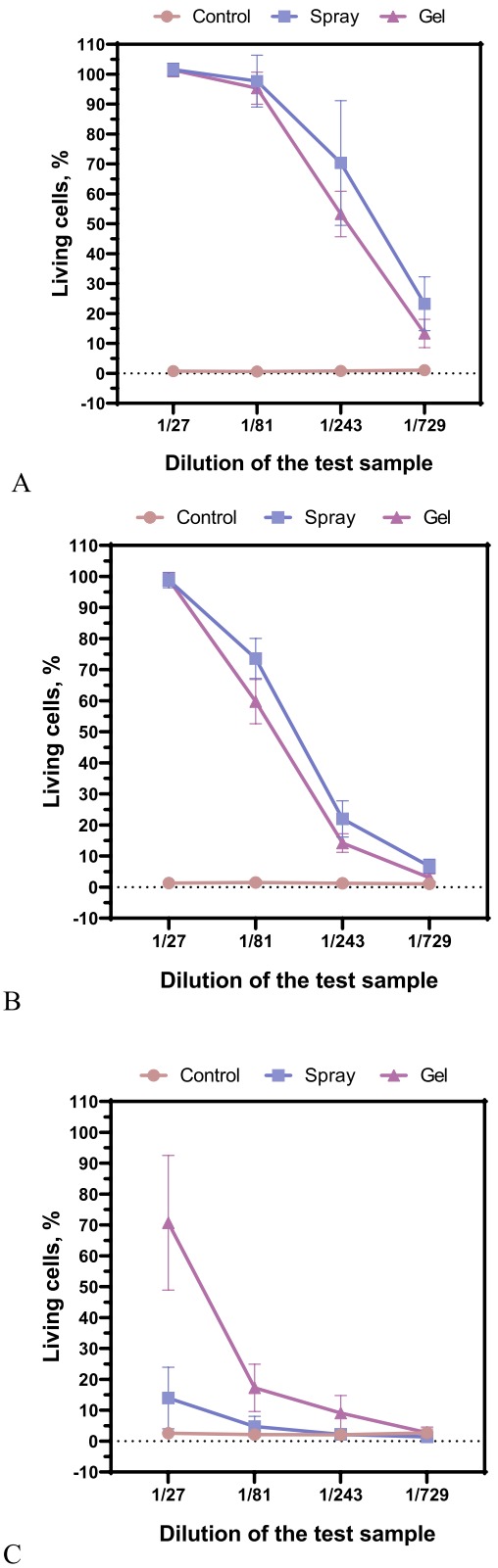

Fig. 2 Antiviral effect of spray and gel. Viability of MDBK cells in vitro: **A** 1 h of action of the test drug; **B** 3 h of action of the test drug; **C** 4 h of action of the test drug

administration of physiological solution) revealed the absence of IFN at all time points of the experiment (Figs. 2, 3).

These findings support the long-term specific antiviral activity of the studied preparations of nasal IFN: the biological effect, expressed in the antiviral characteristic, persists for a long period (4 h) when the drug is in the nasal cavity (longer time was not investigated according to the chosen design of the experiment). Moreover, WNC after 4 h the administration of the IFN/gel contained a statistically significant higher amount of IFN (by 4 times) compared to IFN/spray.

Discussion

Intranasal drug delivery for antiviral agents has been studied and used for many years, as this method of drug administration has the potential for the prevention and treatment of viral infections spread by airborne droplets, particularly and SARS-CoV-2 [11]. Notably, the oral method of taking drugs is considered the most common, but requires certain criteria for the drug, in particular, APIs are water-soluble. Moreover, the advantages of the nasal method of drug administration are the ease of penetration through the mucous membrane with low enzymatic activity. Today, a new direction for nanodelivery of APIs through the nasal method of administration (using liposomes, microspheres, etc.) is being actively researched and developed, especially from the point of view of nanopreparations of vaccines, antigens, antiviral agents, etc. It is important to use adequate carriers to overcome certain disadvantages, particularly degradation [12]. The search for effective and safe drugs against COVID-19 has led to major drug repurposing efforts, with the speed of their discovery being extremely important, which often led to accidental use and was not necessarily supported by established evidence of effectiveness against COVID-19 but relied on the experience of previous epidemics. However, the efficacy of these approaches has often been low, as demonstrated by the WHO Solidarity and Recovery study, which may be due to nonspecific activity against SARS-CoV-2, potentially incorrect timing of administration, and side effects due to systemic administration, which, in addition, cannot provide effective concentrations in infected cells. [13–16]. At the same time, according to the authors, local administration of even low doses of antiviral agents into the upper respiratory tract provides high concentrations of the drug at the site of ongoing virus replication, limiting systemic exposure and unwanted side effects [7]. Therefore, further development and evaluation of the safety and effectiveness of antiviral agents with powerful local action are needed. In this case, the use of IFN is extremely promising from the standpoint of the long-known safety and

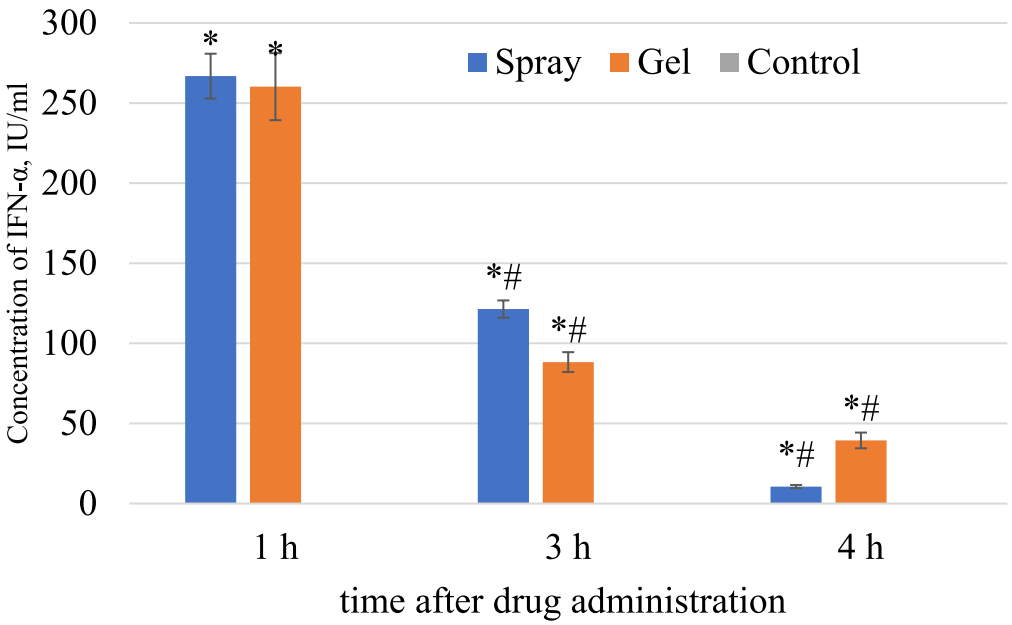

Fig. 3 The average value of the indicators of antiviral activity (IFN concentration) in vitro of WNC samples after intranasal administration of the IFN spray and gel. **Note*: Changes in indicators are statistically significant relative to the control group, $p \leq 0.05$; # Note: Changes in indicators are statistically significant relative to each other, $p \leq 0.05$

effectiveness profile of this medicinal product, low toxicity and significant antiviral activity; its contraindications and other necessary information for clinical use are known. Moreover, the time of local action of the drug is extremely important, which was significantly increased while maintaining antiviral activity by using a new form of nasal spray with the innovative composition of the drug, which is based on a colloidal solution — a sol that turns into a gel after spraying in the nasal cavity: the composition of the drug contains a combination of high-molecular compounds that form a gel with thixotropic properties, which, under the influence of mechanical forces turns into a liquid sol and forms a gel again in a state of rest. It was shown that IFN nasal preparations in the form of a spray and gel retain their specific (local) antiviral activity when in the nasal cavity of experimental animals for 4 h (a longer period was not investigated). The highest concentration of IFN in the WNC of the rats was observed 1 h after the drug was administered, while the drug both in the form of a spray and a gel showed similar results in terms of biological activity (antiviral) in the washings. In the WNC of the rats after the introduction of the nasal IFN in the form of a gel, a statistically significantly higher amount of IFN (4 times greater) was observed at 4 h after administration, than the amount of the cytokine in the form of a spray, which indicates a longer local effect with preservation of antiviral activity.

In addition, we developed an algorithm for evaluating the duration of specific (antiviral) activity of interferons in a complex analysis using model systems in vivo and in vitro, which is quite simple, fast, but at the same time adequate and informative. One of the significant advantages of such an algorithm is that such a system for evaluating the antiviral activity of nasal drugs allows one to study the duration of the local antiviral effect of drugs. However, the use of a sensitive cellular test system to determine the biological activity of IFN is another advantage of this assessment method, as it provides information not only about its presence (as assessed by ELISA). Importantly, the evaluation of the activity of the drug in the dynamics does not involve any serious manipulations with animals that require necropsy for the study of the mucosa, or similar manipulations and can be widely used by various laboratories for the preclinical evaluation of the activity of new antiviral drugs.

Conclusion

After the coronavirus disease 2019 (COVID-19) pandemic, the development of effective and, at the same time, safe, easy-to-use antiviral agents that can exert antiviral activity locally for as long as possible, which will provide a barrier function and, accordingly, reduce the possibility of the virus entering, remains in demand. In summary, we created an algorithm for estimating the duration of antiviral (local) activity. The developed system for evaluating the antiviral activity of nasal preparations allows for studying the dynamics of the duration of the local antiviral effect of the IFN. With the use of such

an algorithm, we showed that the antiviral activity of the studied IFN nasal preparations (in the form of spray and gel) was preserved for 4 h after intranasal administration. Moreover, the concentration of IFN in the form of a gel on the walls of the nasal cavity 4 h after administration was significantly greater than that of IFN in the form of a spray.

Abbreviations

API	Active pharmaceutical ingredient
IC50	Cytotoxicity index
IFN	Interferon α-2b
IEPOR	R.E.Kavetsky institute of experimental pathology, oncology, and radiobiology
IU	International units
NCS	Newborn calf serum
VSV	Vesicular stomatitis virus
WNC	Washed from the nasal cavity

Author contributions

N.B. designed the experiments and wrote the manuscript. O.L. provided feedback on the study, analysis of data and checked the data and all manuscript during submission. G.B, Ye.K. and M.B. helped in writing the manuscript and data analysis.

Funding

The work was carried out within the framework of the project "Study of the specific activity of "Nazoferon Forte" in experimental model systems", contract No. 151-442/21.

Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare that they have no potential competing interests with respect to the research, authorship, or publication of this article.

Received: 5 January 2025 Accepted: 20 February 2025
Published online: 10 April 2025

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