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Study of Dosage-Dependent Effects of Cytostatic Drugs Using a Fibroblast Cell Culture of the Human Nasal Mucosa

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Abstract

Introduction Knowing a concentration at which cytostatic drugs are toxic for the nasal fibroblasts will enable the use cytostatic drugs in the clinical practice to prevent excessive cicatrization.

Objective To determine the cytostatic concentrations of mitomycin C, doxorubicin, and 5-fluorouracil affecting nasal mucosa fibroblasts.

Methods We obtained material during an endonasal dacryocystorhinostomy with the patient's informed consent. The cells were cultivated. Second- to fourth-passage cells were used in the experiments. The cells were stained for vimentin and cluster of differentiation 90 (CD90). An MTS test 3 (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); cell viability test was performed.

Results The cytostatic drugs have a toxic effect on cultivated fibroblasts of the nasal mucosa. This effect is dose-dependent. In terms of reducing the level of tissue fibrotisation in the nasal cavity, the most justified approach is to carry out an experimental study of the effect of mitomycin C, doxorubicin, and 5-fluorouracil at the concentrations of 0.25 mg/ml, 0.25 mg/ml, and 12.5 mg/ml respectively.

Conclusion The authors argue that it is inappropriate to use these cytostatic drugs to conduct studies with the goal of analyzing their antifibrotic effect on the nasal mucosa at concentrations that are either lower or higher than the aforementioned ones.

Keywords
- fibroblasts
- fibrosis
- mitomycin C
- doxorubicin
- 5-fluorouracil
- cytostatic drugs

Introduction

Dacryocystorhinostomy is one of the most widespread surgeries performed on patients with primary acquired nasolacrimal duct obstruction. The purpose of this surgical intervention is to form stable epithelized anastomosis (ostium) between the lacrimal sac cavity and the nasal cavity. Despite improvements in the surgical technique, data show that the rate of recurrences of this intervention is as high as 13 to 17%.[1,2] One of the most common causes for a negative surgery outcome is excessive cicatrization at the ostium site.[3,4] Recently, cytostatic drugs, which are administered either applicationally or by injection into the mucous membrane of the nasal cavity or lacrimal sac at the final stage of the surgery, have become relatively

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widespread to reduce the severity of the fibrotization. Cytostatic drugs are known to reduce fibroblast proliferation, which, in turn, causes a reduction in fibrotization in the region in which a cytostatic drug is administered.[5]

Several authors have reported an accentuated decrease in the number of lacrimal passage obliteration recurrences following the administration of cytostatic drugs,[6,7] while other authors did not report this effect.[8,9] The authors of the present study argue that the absence of clinically-significant fibrotization inhibition was due to the fact that the required cytostatic concentration had not yet been achieved in the tissues of the formed anastomosis region.[8] The present paper is a study on the dosage-dependent effect that cytostatic drugs have on cultivated fibroblasts of the human nasal mucosa. Knowing a concentration at which cytostatic drugs retain their toxicity against nasal mucosa fibroblasts will enable the implementation of these techniques in the clinical practice to prevent excessive cicatrization when performing various surgical interventions in the nasal cavity, particularly dacryocystorhinostomy.

The purpose of the present paper is to determine the cytotoxic concentrations of mitomycin C, doxorubicin and 5-fluorouracil that affect the nasal mucosa fibroblasts.

Methods

Isolation and Characterization of the Cell Culture

The authors obtained the histological material necessary for the study during an endonasal endoscopic dacryocystorhinostomy. Following nasal mucosa decongestion with 0.1% xylometazoline solution and local topical anesthesia with 10% lidocaine solution (Pharmstandard-Leksredstva, Kursk, Kursk Oblast, Russia), a 1.5-mm deep horizontal incision (using a diamond-shaped knife with an incision depth limitation) was made in the lacrimal fossa projection region, and 4×2-mm section of mucous membrane was bluntly separated at that depth. This biopsy specimen was soaked in 0.04% gentacimin solution (Dalhimfarm, Khabarovsk, Khabarovskiy kray, Russia) for 30 minutes, and then transferred to the cell technology laboratory for further treatment.

The specimen was sliced into 1×1-mm fragments using a surgical scalpel. The fragments were placed in Petri dishes with a diameter and growth surface area of 3.5 cm and 10 cm^2 respectively (Corning, Corning, NY, US) and cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 2 mM of glutamine, 100 U/ml of penicillin, 0.1 mg/ml of streptomycin (Gibco, Thermo Fisher, Waltham, MA, US) and 10% Fetal bovine serum (FBS) at 37°C, in a humidified atmosphere containing 5% of CO_2. On the 4th to 5th days of cultivation, the cells started migrating from the fragment tissue to the plastic. By the 14th day of cultivation, the explants were removed from the dishes, and the remaining cells were dissociated with a 0.05% Trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Gibco) and passaged in T25 culture flask. Our previous experience with explant cultivation showed that the proliferation of first-passage fibroblasts is not sufficiently active. Due to this, a growth factor was added to the first passage medium. First-passage cells were cultivated in DMEM using the aforementioned composition and fibroblast growth factors (FGF, Sigma-Aldrich, St. Louis, MO, US) with a concentration of 4 ng/ml, while the second-passage cells had no FGF treatment. The growth medium was replaced every 3 to 4 days. The cells were subcultured at a 1:4 to 1:6 ratio. Cell-growth monitoring and the morphology assessment were performed using a Zeiss Axio Vert.A1 (Carl Zeiss, Oberkochen, Germany) inverted microscope. Second- to fourth-passage cells were used in the experiments.

Immunocytochemical Analysis

To characterize the cell culture obtained, the cells were stained for fibroblast specific markers: vimentin and cluster of differentiation 90 (CD90). The cells were grown in Petri dishes for confocal microscopy (5×10^3 cells per dish). After attaining 50% of confluence, the cells were fixed with 4% paraformaldehyde (10 minutes at 4°C), washed 3 times with Phosphate buffered saline (PBS), and incubated for 30 minutes at room temperature in PBS containing 0.2% tween-20, 0.2% triton x-100, and 2% goat serum. Then the samples were incubated with primary antibodies to vimentin (1:40; Abcam, Cambridge, United Kingdom,) in PBS with 0.2% tween-20 and 0.2% goat serum (for 1 hour at 37°C), washed 3 times with PBS, and incubated with second anti-mouse immunoglobulin antibodies (goat anti-mouse Alexa Fluor 555, 1 µg/ml, Invitrogen, Carlsbad, CA, US) for 1 hour at 37°C. The samples were washed with PBS, and the cell nuclei were poststained with 4',6-diamidino-2-phenylindole (DAPI) (1:400; Invitrogen). For CD90 visualization, the cells were incubated for 10 minutes at 4° C with anti-CD90 primarily labeled antibodies (1:11; Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Gernamy) carrying phycoerythrin fluorescent dye (►**Fig. 1**).

Confocal Laser Scanning Microscopy

The scanning was performed using a A1R MP+ confocal laser scanning microscope (Nikon, Shinagawa, Tokyo, Japan). The 405-nm and 561-nm emission lasers and the following optics were used in the present study: Plan Apo 20x/0,75 Dic N, Apo IR 60x/1,27 WI and Apo TIRF 60x/1,49 oil Dic lens (Nikon, Shinagawa, Tokyo, Japan). The cell contours were visualized using differential interference contrast. The images obtained were processed using the NIS-Elements AR software (Nikon).

Cell Viability Assay

the CellTiter 96 AQueous One Solution Reagent kit (Promega, Madison, WI, US) was used for the MTS test. The MTS reagent was defrosted immediately before use. The test was performed according to manufacturer's instructions (procedure TB245). The cells were cultured in 96-well plates (3,000 cells/well) in DMEM containing 10% FBS, antibiotics (100 units/mL of penicillin, 100 µg/ml of streptomycin, Gibco), and GlutaMax (2 mM, Gibco). After 24 hours of cultivation, drugs at various concentrations obtained by serial dilutions in the growth medium were added inside the wells. After 24 hours of incubation, 20 µl of MTS (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-

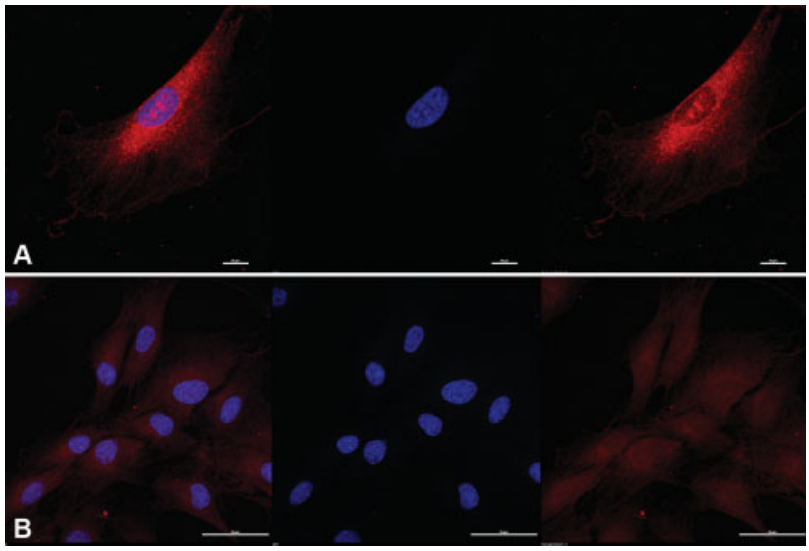

Fig. 1 Immunocytochemical staining. Confocal laser scanning microscopy (Nikon A1R MP+). Cell nuclei are stained with DAPI (1:400; Invitrogen) (blue fluorescence). Bar scale: 50 µm. (**A**) Anti-vimentin primary monoclonal antibodies (1:40; Abcam) and antimouse immunoglobulin secondary antibodies, conjugated with Alexa555 (1:750; Invitrogen) fluorescent dye (red fluorescence). (**B**) Primarily labeled anti-cluster of differentiation 90 - phycoerythin antibodies (1:11; Miltenyi Biotec) for CD90 visualization (red fluorescence).

tetrazolium) reagent were added to each well containing 100 µl of growth medium. The cells incubated with the culture medium were used as the negative control. The culture medium without cells was used for the blank.

The optical density was measured at 490 nm after 4 hours of incubation with the MTS reagent. The cell viability was calculated using the following formula:

Number of living cells $= (A_s - A_b / A_c - A_b) \times 100\%$

A_s – mean value of the specimen's optical density (OD); mean OD sample;

A_b – mean value of the blank sample's optical density; mean OD control blank;

A_c – mean value of the control's optical density; mean OD control.

Values of IC50 were found with Hill equation using the GraphPad Prism 6 (GraphPad Software, San Diego, CA, US) software.

Results

As a result of the study, a human nasal mucosa fibroblast cell culture positive for specific markers (vimentin and CD90) was obtained.

The culture was used as a test system to determine the cytotoxicity effect of the following cytostatic drugs: mitomycin c, doxorubicin and 5-fluorouracil. The MTS test is a colorimetric method that enables the determination of the number of viable cells when studying cell proliferation and the cytotoxicity of various drugs. Mitochondrial NADH nicotinamide adenine dinucleotide (reduced)-dependent oxidoreductases are capable of reducing the MTS reagent to formazan, whose absorption rate reaches its maximum value at 490 nm to 500 nm.

►**Table 1** shows the results of the analysis of the number of viable fibroblasts depending on the concentration of the cytostatic drugs used. These data are shown in ►**Fig. 2**. The IC50 values for mitomycin c, doxorubicin and 5-fluorouracil were 113 mkg/ml, 392 mkg/ml and 19 mkg/ml respectively.

Discussion

The present paper is a study on the effect of cytostatic drugs on cultivated fibroblasts of the nasal mucosa. The cytostatic drugs studied are known to reduce fibroblast proliferation by inhibiting DNA replication. The effect of doxorubicin is intercalation, that is, integration of the nitrogen bases of the DNA, which inhibits the effect of topoisomerase II, thus making the relaxation of super-spiralized DNA sections impossible, and disturbing the transcription process. The effect of 5-fluororacil implies replacing uracil with fluorouracil in a replicated RNA molecule, which make its further processes impossible. The effect of mitomycin C leads to the formation of covalent bonds between complimentary DNA strands, thus hampering its replication.

The use of mitomycin C for antifibrotic purposes has significantly increased in the lacrimal surgery practice.[5–9] There are also several reports on the use of 5-fluorouracil as an antifibrotic agent for dacryocystorhinostomy.[10–12] The authors found no evidence of the use of doxorubicin to prevent dacryocystitis recurrence. However, in vitro studies regarding mucosal cells showed the effect of doxorubicin on collagenogenesis.[13]

As the analysis of the results of the present study showed, the toxic effect that the drugs in question had on the cultivated fibroblasts of the human nasal mucosa was dosage-dependent.

The present study showed that the toxic effect that mitomycin C had on fibroblasts of the human nasal mucosa was sufficient for to terminate fibroblast growth at a concentration of 0.25 mg/ml, which corresponds to the data of a previous research.[5]

To date, no studies on the toxicity of doxorubicin and 5-fluorouracil against fibroblast cultures of the nasal mucosa have been performed. In the present study, the authors found

Table 1 Results of the cytotoxicity analysis

Mitomycin C				Doxorubicin				5-fluorouracil				
Concentration (mg/ml)	Survival (%)		Mean ± standard deviation	Concentration (mg/ml)	Survival (%)		Mean ± standard deviation	Concentration (mg/ml)	Survival, (%)			Mean ± standard deviation
	Test 1	Test 2			Test 1	Test 2			Test 1	Test 2	Test 3	
1	0	0	0 ± 0	1	0	0	0 ± 0	25	0	14	0	5 ± 8
0.25	14	17	16 ± 2	0.25	33	46	39 ± 9	12.5	2	27	51	27 ± 25
0.006	53	55	54 ± 2	0.006	51	78	65 ± 19	6.25	63	67	60	64 ± 4
0.015	59	62	61 ± 2	0.015	71	77	74 ± 4	3.125	75	78	75	76 ± 2
0.003	71	72	71 ± 1	0.003	79	84	82 ± 3	1.5625	78	80	78	79 ± 1
0.0009	81	94	87 ± 9	0.0009	86	83	85 ± 2	0.7813	81	84	78	81 ± 3

that the optimal effective doxorubicin concentration for the termination of fibroblast growth is 0.25 mg/ml; yet, at this concentration, its toxic effect on the fibroblasts is smaller than that of mitomycin C at the same concentration. Thus, it is plausible to expect a lower clinical effect of doxorubicin at the concentration of 0.25 mg/ml, when compared to that of mitomycin C at the same concentration and dosage.

The analysis of the toxic effect of 5-fluorouracil showed that 12.5 mg/ml is the optimal concentration of this drug to terminate fibroblast growth; however, at this concentration its toxic effect on nasal cavity fibroblasts is also lower than that of mitomycin C when used at the concentration of 0.25 mg/ml.

In all cases, an increase in concentration made the toxicity of the drugs studied get close to its absolute value, while a decrease inhibited the cell's viability to a degree apparently insufficient for the development of the clinical effects.

Surgeons currently tend to use cytostatic drugs at empirically chosen concentrations, which leads to negative outcomes and deviation from the technique: for example, in the studies by Bakri et al[11] and Watts et al,[12] the authors used 5-fluorouracil at concentrations of 0.5 mg/ml and 25 mg/ml respectively. The present study provides a clue to explain the reason behind the lack of effect of the drug in the former study and the relatively poor results in the latter one.

The results obtained in the present study enable us to assume that the use of cytostatic drugs at exactly the concentrations determined by the authors may enable the achievement of the maximum antifibrotic effect, with a simultaneous reduction in the number of undesirable side effects on the cells, which also affects postdacryocystorhinostomy tissue regeneration processes. The authors argue that it is appropriate to use these data when carrying out experiments using a more complex model. Regarding the fact that the in vitro research could not be directly extrapolated to the clinical practice, we suppose that an experimental trial with an animal model should be an appropriate following step for the present research. The data obtained with an animal model-based research could be translated into the clinical practice.

Conclusion

The cytostatic drugs studied have a toxic effect on cultivated fibroblasts of the nasal mucosa. The authors showed that this effect is dosage-dependent. In terms of reducing the level of tissue fibrotization in the nasal cavity and, particularly, in the dacryocystorhinostomy-formed junction site, the most justified approach is to carry out an experimental study on the effect of mitomycin C, doxorubicin and 5-fluorouracil at the concentrations of 0.25 mg/ml, 0.25 mg/ml, and 12.5 mg/ml respectively.

The authors suppose it is inappropriate to use these cytostatic drugs to conduct studies with the goal of analyzing their antifibrotic effect on the nasal mucosa at concentrations that differ from the aforementioned ones, since at such concentrations the drugs either cannot have a clinically-significant effect or, as the authors assume, can have an undesirable effect by inhibiting the proliferation not only of fibroblasts, but also of other cells affecting nasal mucosa reparation.

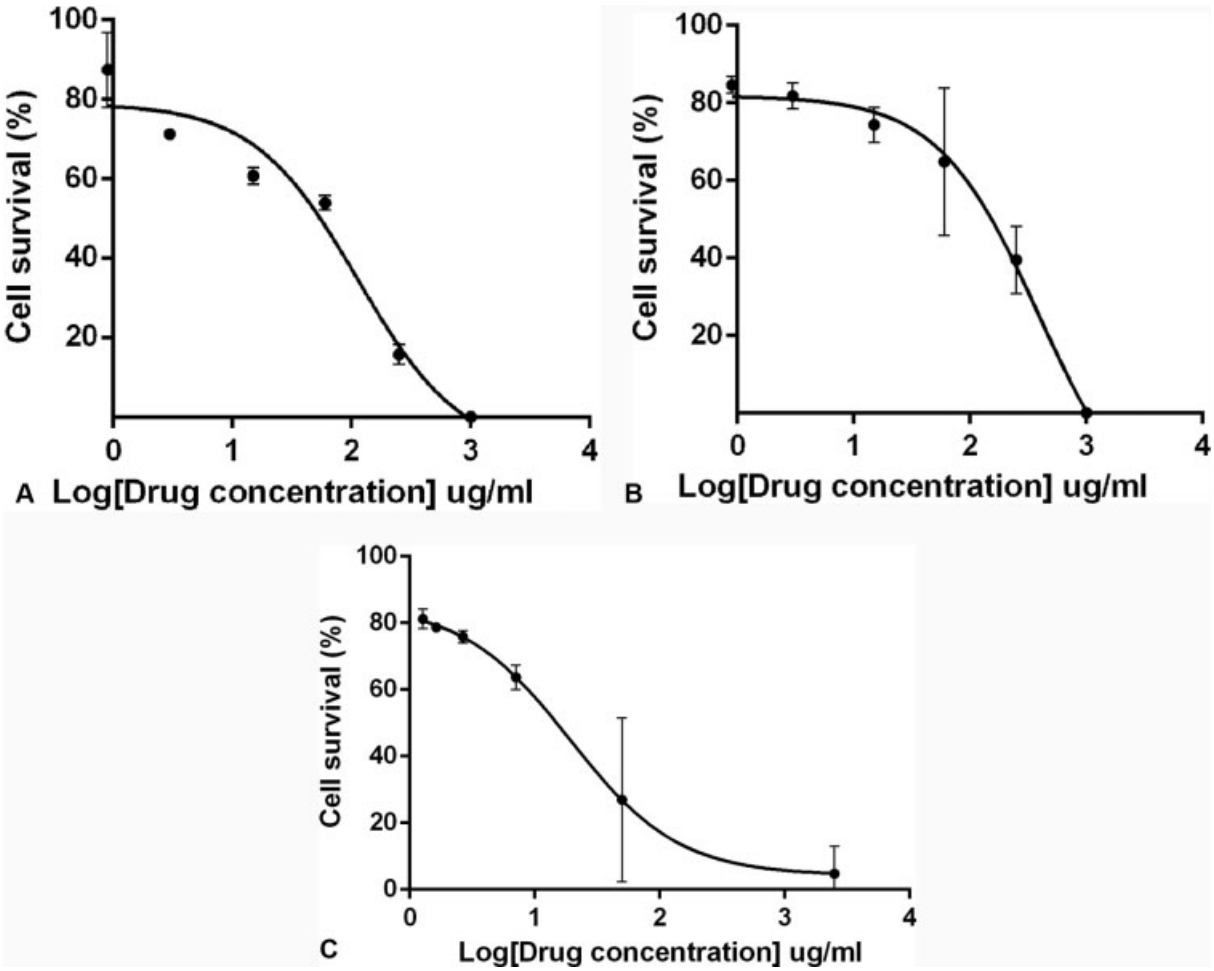

Fig. 2 Cell viability and drug concentration. (**A**) Mitomycin C; (**B**) doxorubicin; (**C**) 5-fluorouracil.

Conflict of Interest

The authors have none to declare.

All procedures performed in the present study were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its amendments. The present research was approved by the local Ethical Committee of Scientific Research of the Institute of Eye Diseases.

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