ORIGINAL PAPER
DOI: 10.5603/OJ.2021.0007

Modeling of bacterial keratitis of *Pseudomonas aeruginosa* etiology in the experimental model

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ABSTRACT

BACKGROUND: *Pseudomonas aeruginosa* keratitis demonstrates an aggressive course, high resistance to antimicrobial therapy, and it also leads to a significant reduction of visual acuity. The purpose of our study was to develop an experimental model of *Pseudomonas aeruginosa* keratitis and investigate clinical features of corneal lesions in rabbits.
MATERIAL AND METHODS: A total of 32 rabbits were divided randomly into three groups. The animals of the experimental group 1 (8 rabbits) underwent scarification of the cornea and instillation of archival strain suspension of *P. aeruginosa* into the conjunctival sac. In group 2 (12 rabbits), corneal de-epithelialization followed by instillation of microbial suspension was performed. In animals of group 3 (12 rabbits), after the de-epithelialization followed by instillation of one-day suspension of the pathogen, the cornea's surface was covered with a sterile soft contact lens for 16–24 hours. In half of the animals (6 rabbits), the contact lenses with bacterial films of *P. aeruginosa* were used as a modification.
RESULTS: The technique used in group I resulted in bacterial conjunctivitis with mild corneal changes in all cases. In group II, keratitis development was noted in all animals, being defined as moderate in nine cases and semi-severe in three. In animals of group III, severe purulent keratitis occurred in 11 of 12 eyes and semi-severe — in one. Four cases of ulcers and two cases of corneal abscess were recorded, resulting in perforations and keratomalacia (2 cases each).
CONCLUSIONS: Modeling of purulent *P. aeruginosa* keratitis in rabbits of varying severity requires complete de-epithelialization cornea segment and prolonged presence of the pathogen on the affected surface. The effective way to keep infectious organisms on the cornea surface in sufficient concentration is to use soft contact lenses.

KEY WORDS: purulent keratitis; *Pseudomonas aeruginosa*; experimental model; rabbits

Ophthalmol J 2021; Vol. 6, 17–22

INTRODUCTION

Despite modern achievements in the treatment of inflammatory diseases of the cornea, bacterial keratitis is still associated with the danger of visual loss, leading to permanent disability and requiring significant expenses for treatment and rehabilitation [1, 2].

In recent years, many authors have noted some changes in the risk factors for bacterial keratitis, as well as the range of pathogens causing them. For instance, keratitis in contact lens wearers can reach 44–65% among all cases of the disease [3–5]. Gram-positive bacteria have been traditionally associated with the development of bacterial keratitis. However, over the past decade, gram-negative flora proved to cause bacterial lesions of the cornea as well. The most common of those organisms is *Pseudomonas aeruginosa*, as it is isolated in 22–40% of patients with bacterial keratitis [3, 5, 6]. It was determined as gram-negative causative agent of corneal ulcers No. 1 (CDC, USA, 1990-1996) [7].

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Purulent corneal lesions caused by *P. aeruginosa* are characterized by aggressive course, high resistance to antimicrobial therapy, and lead to a significant reduction of visual acuity. Quite often, there is a risk of corneal perforation and endophthalmitis, which may result in loss of an eye itself [3, 5, 8]. Therefore, this problem is of great social significance, which requires further study.

Creation of experimental animal models of bacterial keratitis is considered to be an effective method to investigate its morphology, pathogenesis, and microbiology, prompting the development of novel treatment regimens and correction of existing ones, as well as establishing relevant prognostic criteria depending on the severity of the disease [9–11].

The purpose of our study was to develop an experimental model of *Pseudomonas aeruginosa* keratitis and investigate clinical features of corneal lesions in rabbits.

MATERIAL AND METHODS

The study was performed at the Scientific and Biological Clinic (vivarium) of National Pirogov Memorial Medical University, Vinnytsya, during 2019–2020. The experiment was conducted in compliance with ethical requirements of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986), the norms of biomedical ethics adopted by I National Bioethical Congress (Kyiv, 2001), as well as according to the Law of Ukraine No. 3447-IV "On protection of animals from cruel treatment" (Kyiv, 2006). The study was reviewed and approved by the Bioethics Commission of National Pirogov Memorial Medical University, Vinnytsya (Ukraine), on October 27, 2016 (Record No 10).

A total of 32 rabbits were included in the study (adult animals of 3–3.5 kg). Animals were kept in separate cages throughout the study period on a standard light/dark cycle (12/12 h), being provided with the natural food and water *ad libitum*. Modeling of keratitis was performed in three experimental groups under aseptic conditions and general anesthesia (using sodium thiopental 0.1% — 1 mL per kg), with additional epibulbar anesthesia (instillation of sol. alcaini 0.5%). Keratitis was modeled on one of the eyes of each animal. An archival strain of *Pseudomonas aeruginosa* obtained at the Museum of Bacterial Cultures of Bacteriological Laboratory (National Pirogov Memorial Medical University, Vinnytsya) was used for inducing infection in the form of suspension of a one-day culture of the microorganism in concentration 5×10^8 CFU/mL, which corresponds to the 0.5 McFarland standard. All animals were divided randomly into three groups.

The animals of experimental group 1 (8 rabbits) underwent scarification (making five linear scratches 5 mm long) in the center of the cornea and instillation of 0.2–0.4 mL of a suspension of the pathogen into the conjunctival sac with its additional subconjunctival administration (0.1 mL).

In experimental group 2 (12 rabbits), corneal deepithelialization was made in the central part involving the area of about 1 cm2 by scraping of its surface layer with the tip of a 21 G injection needle. The microbial suspension was instilled on the surface of the eye.

In animals of group 3 (12 rabbits), after de-epithelialization and instillation of one-day microbial suspension (0.2–0.4 mL), the surface of the cornea was covered with a sterile soft contact lens made from balafilcon A (water content: 36%, oxygen permeability DK/t: 110.0) for 16–24 hours. In half of the animals (6 rabbits), the contact lenses with bacterial films of *P. aeruginosa* were used as a modification. For this purpose, sterile lenses were contaminated in broth culture of *P. aeruginosa* strain (concentration of cells 10^6–10^7 per 1 mL) with subsequent incubation at 37°C for 48 hours until the appearance of microbial biofilm on the surface of lenses.

Initial assessment of bacterial lesion of the cornea was conducted 24 hours after inducing infection. The course of the disease was monitored daily by evaluation of clinical signs of keratitis, taking material for culturing, and performing the ophthalmological examination of the cornea using a fluorescein eye stain test and photo fixation. Clinical assessment of eye lesion severity was done using the Draize scoring system [12].

Microbiology specimen collection was performed with a sterile swab every 24 hours starting from the time of bacterial contamination to the moment of withdrawal of animals from the study.

Animals were withdrawn from the experiment under aseptic conditions by air embolism (1–1.5 cm^3) through the auricular vein with subsequent collection of the cornea for microbiological and histological studies. It was done at the height of the inflammatory process after discontinuation of *P. aeruginosa* inoculation (6^{th}–7^{th} days), as well as af-

ter subsidence of inflammation and corneal surface epithelialization confirmed by negative fluorescein test (mainly on 12th–14th days). In the most severe cases, when corneal epithelialization did not occur, the animals were withdrawn on the 14th day. The animals with perforations of corneal abscess were withdrawn on the same day. Thus, it was possible to investigate the bacteriological and pathomorphological characteristics of the process at various stages of its development.

Statistical processing of obtained data included calculation of descriptive characteristics using STATISTICA 6.0 program and comparative assessment of descriptors for a number of evaluation parameters.

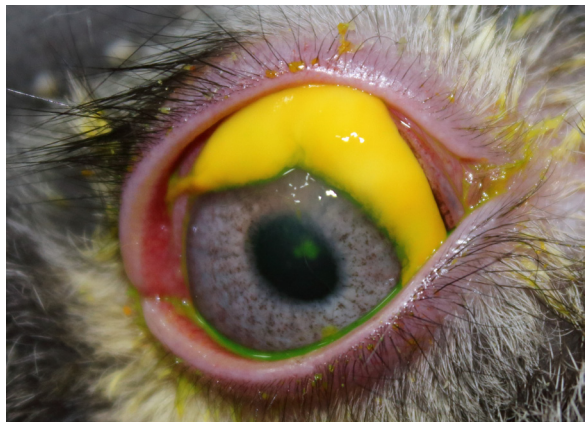

FIGURE 1. Conjunctival abscess; erosion in scarification area visible after fluorescein staining

RESULTS

The severity of bacterial inflammation of the ocular surface in rabbits differed between experimental groups. Infecting with *P. aeruginosa* and linear scratching of the corneal surface in group 1 led to bacterial conjunctivitis with no persistent purulent inflammation of the cornea. Twenty-four hours after inducing infection, all animals of this group developed conjunctivitis, manifested by severe (5 eyes) or moderate (3 eyes) conjunctival hyperemia and third eyelid swelling, lacrimation. Three of eight rabbits demonstrated purulent thick white discharge from the eyes while the remainder — discharge in the form of fibrin threads. Corneal changes in all cases were minimal: in the scarification area, there were erosions, almost invisible during the ophthalmological examination. A local, relatively well-defined stained area of a smaller size than scarification area was observed when using fluorescein. Spontaneous epithelialization of such erosions occurred within 3–4 days. Inflammation of conjunctiva persisted for the following 24 hours and gradually decreased up to the 5th day of the experiment. Pure culture of *P. aeruginosa* was obtained from the conjunctiva for two days in all cases. Mixed flora (*P. aeruginosa* + *St. aureus*) was discovered on the 3rd day. On the 4th day, *P. aeruginosa* culture was not obtained. In one case, a conjunctival abscess was formed at the site of subconjunctival injection of microbial suspension, which ruptured on the 5th day (Fig. 1). Bacteriological examination of abscess content revealed a pure culture of *P. aeruginosa*.

All animals of group 2 who underwent de-epithelialization of the cornea with subsequent application of microbial suspension of *P. aeruginosa* were found to have keratitis. Twenty-four hours after inducing infection, three of 12 rabbits developed profuse white viscous purulent discharge; the remainder had a moderate mucopurulent and fibrinous discharge. Marked hyperemia and swelling of the conjunctiva and third eyelid were observed in all eyes. In all cases, there was a local inflammatory infiltration of the cornea with severe local edema and positive fluorescein test all over the de-epithelialization area. The inflammatory focus was surrounded by a transparent rim. Within 3–4 days, the clinical picture became more severe, signs of keratitis increased. It was manifested by the spread of infiltrate and corneal edema beyond the de-epithelialization area with intense fluorescein staining, but no further than the diameter of de-epithelialized surface in any direction; the rim of transparent cornea around the focus of inflammation narrowed. In nine eyes, corneal opacity was regarded as semitransparent, the iris and the pupil being indistinctly visualized. In three other animals, deeper eye structures were not visualized because of the cornea's severe opacity and edema. The inflammatory process was accompanied by photophobia associated with lacrimation and later dryness of the eye surface in all animals (Fig. 2).

Microbiological tests in animals of the second group found pure cultures of *P. aeruginosa* for the first two days of observation. Subsequently, polymorphic flora (staphylococci, bacilli, molds) joined. On the fourth day, the samples were negative for *P. aeruginosa*, but showed the presence of other microorganisms in small quantities (up to 10 CFU/mL). Studying cornea segments obtained after the withdrawal of animals from the experiment *P. aeruginosa* was isolated in 5 of 12 rabbits despite previous negative culturing results.

FIGURE 2. Moderate keratitis; fluorescein eye stain test

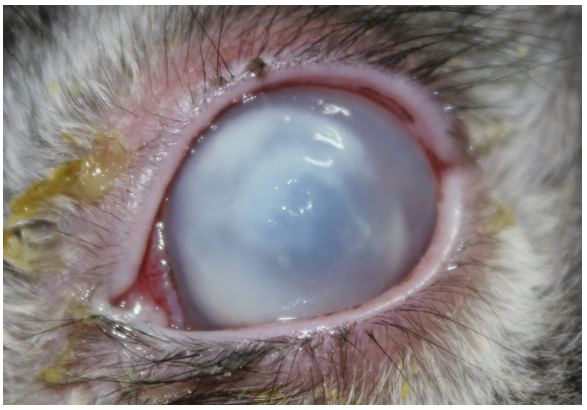

FIGURE 4. Severe keratitis

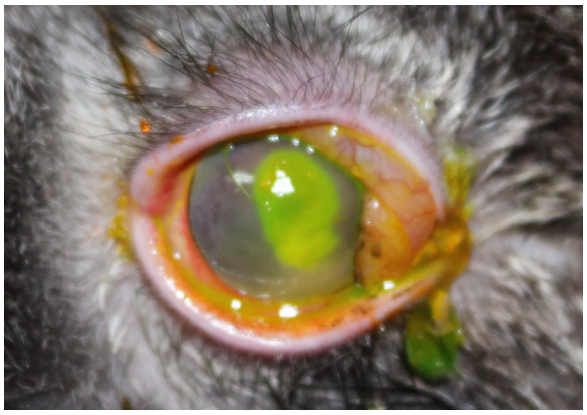

FIGURE 3. Semi-severe keratitis; fluorescein eye stain test

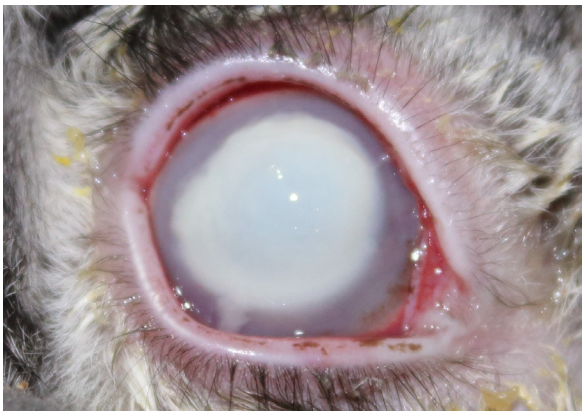

FIGURE 5. Corneal abscess

The development of keratitis was registered in all animals of group 3. The inflammatory process was characterized by a much larger area and depth of lesions. Severe hyperemia of bulbar conjunctiva and third eyelid, pronounced chemosis, profuse purulent discharge associated with sticky eyes were commonly found. The area of corneal opacity significantly exceeded the area of de-epithelialization up to subtotal and total involvement. Because of the high intensity of corneal opacification, deeper structures of the eye were visualized either partially (8 cases) or were not identified at all (4 cases) (Fig. 3, 4).

During the second day of study, there was a progression of the process in all 12 animals of group 3, associated with ulceration in two of them. Instillation of fluorescein caused uneven diffuse impregnation of the cornea by the dye and its penetration into deeper layers and decentralized distribution far beyond the zone of de-epithelialization with no distinct demarcation. Corneal deformity with loss of its spherical shape occurred in four cases, corneal abscess — in two (Fig. 5).

In the group of animals where contact lenses with bacterial films of *P. aeruginosa* strain were used, the highest degree of purulent inflammation was manifested at this stage. Further stabilization and gradual slow regression of the process were commonly observed. Swelling of the conjunctiva and third eyelid, as well as purulent discharges from the eyes, lasted for 4–5 days. Mucosal hyperemia persisted within 10–11 days. The area of corneal opacity and its edema gradually decreased in the direction from the periphery starting from the 6^{th}–7^{th} day while the transparent peripheral "rim" increased. The area of fluorescein staining gradually decreased, its fragmentation occurred. Corneal surface defect epithelialized on the 12^{th}–14^{th} days in 8 cases, but the formation of persistent, intense opacity precluded visualization of deeper structures. In the most severe cases, keratomalacia and perforation of the cornea were registered (2 and 2 cases, respectively). Clinical man-

Table 1. Clinical manifestations of ocular surface lesions in rabbits (n = 32)

Clinical manifestations	Group 1 (n = 8)	Group 2 (n = 12)	Group 3 (n = 12)
In 24 hours	3* (37.5%) 5**(62.5%)	9# (75.0%) 3## (25.0%)	4° (33.3%) 8°° (66.7%)
In 48 hours	1* (12.5%) 7** (87.5%)	3## (25.0%) 7### (58.3%) 2#### (16.7%)	1° (8.3%) 11°° (91.7%), incl. 2°°°, 1°°°°
4th day	7* (87.5%) 1***(12.5%)	9###(75.0%) 3####(25.0%)	1° (8.3%) 11°° (91.7%), incl. 4°°°, 2°°°°
Resolution	8 (100%)	1 (8.3%)	
Epithelialization of the cornea without opacity			–
Partial corneal opacity (*nubecula*)	–	8 (66.7%)	1 (8.3%)
Complete corneal opacity (*leucoma*)	–	3 (25.0%)	7 (58.3%)
Keratomalacia	–	–	2 (16.7%)
Perforation	–	–	2 (16.7%)

*corneal erosion + moderate conjunctivitis; **corneal erosion + severe conjunctivitis; ***corneal erosion + subconjunctival abscess; #moderate keratitis + moderate conjunctivitis; ## moderate keratitis + severe conjunctivitis;###semi-severe keratitis; ### severe keratitis;°semi-severe keratitis + intensive conjunctivitis; °°severe keratitis + intensive conjunctivitis; °°°corneal ulcer (severe keratitis); °°°°corneal abscess (severe keratitis)

Table 2. The results of bacteriological tests of rabbits' cornea (n = 32)

	Group 1 (n = 8)	Group 2 (n=12)	Group 3 (n=12)
24 hours	P	P	P
48 hours	P	P	P
72 hours	P + mix	P + mix	P
4th day	0 + mix	0 + mix	P + mix
5th day	0 + mix	0 + mix	0 + mix
6th day	–	–	0 + mix
Cornea (after withdrawal)	–	P (n = 4)	P (n = 11)

P — presence of pure culture of *P. aeruginosa*; 0 — absence of *P. aeruginosa*; mix — mixed flora

ifestations of ocular surface lesions in experimental animals of three groups are presented in Table 1.

Microbiological tests in animals of the third group revealed a pure culture of *P. aeruginosa* for three days, followed by migration towards the polymorphic flora. Starting from the 5th day, the causative microorganism was not isolated, and this generally resembles the dynamics of a microbiological picture in animals of the second group. However, the average number of microorganisms in the samples taken 24 hours after the induced infection was 107 times higher in cases with soft contact lenses. The results of bacteriological tests are summarised in Table 2.

DISCUSSION

Despite differences between some features of bacterial keratitis in man and animal, the use of animal models is rather helpful in understanding the disease [9, 11]. Existing models of *P. aeruginosa* keratitis in rabbits generally differ by method of inoculation (topical or intrastromal), depth of corneal lesion (scarification, abrasion, and mechanical removal of epithelium), and sometimes by presence of artificial materials (silk suture, contact lenses) [9].

This study demonstrated the importance of complete de-epithelialization of the corneal segment to be associated with the prolonged presence of pathogen on the affected surface. By contrast, linear scarifications of the cornea followed by *P. aeruginosa* inoculation did not lead to persistent bacterial keratitis, which would require treatment. Obviously, the ocular infection was eliminated by the local bactericidal and regenerative protection systems of the animal.

Complete de-epithelialization of the corneal surface, accompanied by inoculation of *P. aeruginosa*,

led to the prevalence of moderate keratitis in animals within 48 hours. If left untreated, the disease commonly advanced to semi-severe. The use of this method supplemented with contact lenses resulted in early severe keratitis in almost all cases. Such difference in results within second and third groups makes it possible to predict the severity of experimental disease depending on the investigator's aim. Proposed experimental techniques are easy to be carried out and result in repetition of outcomes.

It should be noted that in the animals of experimental group 3, deep intra-stromal penetration of the pathogen was found, being isolated in 11 of 12 segments of the cornea, despite previous negative *P. aeruginosa* cultures from the corneal surface. This phenomenon is entirely consistent with the idea of migration of *P. aeruginosa* into deep corneal tissues due to the action of virulence factors (proteases, exotoxin A) so that bacteria avoid the action of local protective factors and washing with tear [9,10].

Analysis of obtained results allowed to grade experimental keratitis in rabbits according to the lesion area and the presence of destructive changes [13]. Thus, moderate keratitis is the process accompanied by corneal opacity of the area not exceeding the zone of de-epithelialization; deeper eye structures are visualized. In semi-severe keratitis, the cornea's opacity extends beyond the zone of de-epithelialization but not further than its diameter in any direction; the eye's deeper structures are partially visualized through the opacity. In severe keratitis, subtotal or total corneal opacity develops, deeper structures of the eye are not identified; loss of spherical shape of the cornea occurs, as well as abscess formation and corneal perforation in some cases.

By the results of the experiment, two utility model patents were obtained [14, 15].

CONCLUSIONS

1. The proposed models of *P. aeruginosa* keratitis allow to reproduce in the experiment bacterial corneal lesion of varying degrees of severity.
2. Conditions required for modeling of *P. aeruginosa* keratitis in rabbits prove to be complete de-epithelialization of part of the cornea and prolonged presence of pathogen on the affected surface.
3. An effective way to keep infectious organisms on the ocular surface in sufficient concentration is the instillation of microbial suspension on the surface of the eye with subsequent covering with a soft contact lens. Use of soft lenses with the bacterial film of *P. aeruginosa* strain grown on its surface results in more aggressive purulent process demonstrating deep intra-stromal penetration of pathogen.

Conflict of interests

The authors declare no conflict of interest.

Acknowledgments

The study had no sponsorship.

REFERENCES

4. Sereda K, Drozhzhyna G, Gaidamaka T. The effect of different techniques of cryopreserved amniotic membrane transplantation on the repair processes of the cornea on the bacterial keratitis model. Ophtalmology. East Europe. 2016; 6(2): 229–239.
5. Paclyuchenko K, Mogilevskyy S, Tereshchenko Y, et al. Active approaches in the treatment of bacterial keratitis and purulent corneal ulcers. Arch Ukrain Ophthalmol. 2013; 1(1): 120–127.
6. Drozhzhina GI, Ivanova ON, Ostashevsky VL, et al. Severe corneal infections, caused by contact lens wear. Ophthal J. 2016; 6: 38–43.
7. Green M, Apel A, Stapleton F, et al. Risk factors and causative organisms in microbial keratitis. Cornea. 2008; 27(1): 22–27, doi: 10.1097/ICO.0b013e318156caf2, indexed in Pubmed: 18245962.
8. Jin H, Parker WT, Law NW, et al. Evolving risk factors and antibiotic sensitivity patterns for microbial keratitis at a large county hospital. Br J Ophthalmol. 2017; 101(11): 1483–1487, doi: 10.1136/bjophthalmol-2016-310026, indexed in Pubmed: 28336675.
9. Truong DT, Bui MT, Memon P, et al. Microbial Keratitis at an Urban Public Hospital: A 10-Year Update. J Clin Exp Ophthalmol. 2015; 6(6), doi: 10.4172/2155-9570.1000498, indexed in Pubmed: 28540138.
10. Hauzer A.R., Sriram P. Severe Pseudomonas aeruginosa Infections. Tackling the Conundrum of Drug Resistance. Postgrad Med. 2005;117(1):41-48. msvitu.com /archive/2006/april/article-1.php?print=1.
11. Zimmerman AB, Nixon AD, Rueff EM. Contact lens associated microbial keratitis: practical considerations for the optometrist. Clin Optom (Auckl). 2016; 8: 1–12, doi: 10.2147/OPTO.S66424, indexed in Pubmed: 30214344.
12. Marquart ME. Animal models of bacterial keratitis. J Biomed Biotechnol. 2011; 2011: 680642, doi: 10.1155/2011/680642, indexed in Pubmed: 21274270.
13. McClellan S, Jiang X, Barrett R, et al. High-mobility group box 1: a novel target for treatment of Pseudomonas aeruginosa keratitis. J Immunol. 2015; 194(4): 1776–1787, doi: 10.4049/jimmunol.1401684, indexed in Pubmed: 25589066.
14. Kilic BB, Altiors DD, Demirbilek M, et al. Comparison between corneal cross-linking, topical antibiotic and combined therapy in experimental bacterial keratitis model. Saudi J Ophthalmol. 2018; 32(2): 97–104, doi: 10.1016/j.sjopt.2017.10.003, indexed in Pubmed: 29942176.
15. Petrunya AM, Kutajni, M.A MA. [Study of clinical inflammatory signs in a cornea at simultaneous experimental keratitis and conjunctivitis]. Oftalmologicheskij zhurnal [J. Ophthal.]. 2013; 2: 83–88.
16. Vovk IM, Kryvetska NV, Burkot VM, et al. Microbiological grounds for antimicrobial treatment of experimental pseudomonal keratitis. Rep Vinnytsia Nat Med Univ. 2020; 24(1): 114–117, doi: 10.31393/reports-vnmedical-2020-24(1)-21.
17. Malachkova NV, Kryvetska NV, Vovk IM, Kryvetskiy VF. Patent Ukraine 141155. State Patent Office of Ukraine, Kyiv 2020.
18. Malachkova NV, Kryvetska NV, Vovk IM, Kryvetskiy VF. Patent Ukraine 141156. State Patent Office of Ukraine, Kyiv 2020.