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Original article

Modeling of the Purulent-Inflammatory Process in Mice Against the Background of Immunosuppression Using Hydrocortisone and 2, 6, 10, 14-tetramethylpentadecane. In Vivo Research

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Abstract

Purulent complications of wounds of various etiologies are one of the pressing problems of modern medicine. Antibiotic resistance is currently giving impetus to the development of ever newer and more advanced drugs, or the modernization of forms and delivery methods of existing ones.

***The purpose of the study** was to reproduce the purulent wound model in mice and compare immunosuppression methods using hydrocortisone and the drug 2, 6, 10, 14-tetramethylpentadecane.*

***Methods.** Several variants of modeling the development of a purulent-inflammatory process in skin wounds in mice and rats were performed. When conducting an experiment on mice, three groups were divided: 1. Using hydrocortisone as immunosuppression (at the rate of 25 mg/kg for 7 days). Wounds were applied on the second day of drug administration. Based on the results of 14 individuals. 2. Using the drug Pristane as immunosuppression (at the rate of 500 µl intraperitoneally per 1 individual, 1 time). The wounds were applied on the 7th day. Based on the results of 14 individuals. 3. 14 mice were used in the control group - without immunosuppression. Then two types of bacteria were tested as wound-infecting microorganisms: Staphylococcus aureus, a representative of the normal skin microbiota, and Pseudomonas aeruginosa, as the most common type of pseudomonas, causative agents of nosocomial infections. Wound infection was carried out using a mixed suspension of the above 2 bacterial cultures.*

***Results**. We determined the most optimal model of purulent wounds, namely, the variant with the use of immunosuppression with 2, 6, 10, 14-tetramethyl-pentadecane. The use of this preparation allowed to reduce the number of immunosuppress or injections and to obtain a denser biofilm on the wound surface.*

***Conclusion**. Modeling of purulent wound in mice is possible only on the background of immunosuppression, as which can be used preparations 2, 6, 10, 14-tetramethyl-pentadecane and hydrocortisone.*

***Key words**: purulent wound model, immunosuppression, drugs for immunosuppression in animals.*

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Introduction

Infections resulting from traumas and surgical interventions, as well as increasing antibiotic resistance, are among the most significant challenges in modern surgery [1,2]. Wounds during traumatization and the postoperative period are typically colonized by aerobic and anaerobic bacteria and fungi. These microorganisms are mostly from the resident microbiota of the surrounding skin, oral cavity, and intestine, or from the external environment. They form polymicrobial communities known as biofilms, which are particularly common in chronic wounds [3]. Animal modelling is used to investigate different drugs and their delivery methods, as well as modernisation in the preclinical research phase for various processes, including purulent and inflammatory processes [4]. Developing new therapies for successful wound healing always requires a satisfactory animal model [5]. In a number of preclinical studies, researchers have tested several methods of inducing multispecies infection using known bacterial species from endogenous or exogenous sources.

The purpose of the study was to reproduce the purulent wound model in mice and compare immunosuppression methods using hydrocortisone and the drug 2, 6, 10, 14-tetramethylpentadecane.

Materials and Methods

A randomized in vivo clinical trial was performed at the National Center for Biotechnology. It was conducted on 42 mice randomly distributed into two groups. Inclusion criteria: female white mice of CD4 breed weighing 22+/-0.5g (NCB vivarium, Kazakhstan). Exclusion criteria: males, underweight individuals (weight less than 21.5 grams). Animal housing conditions corresponded to the standard conditions of the vivarium. Air temperature was maintained at 20-24°C, humidity - 45-65%, light regime - 12h light/12h darkness.

Mice were kept in individual cages with free access to food and water. Experiments on animals were conducted humanely in full compliance with the laws and regulations of the Republic of Kazakhstan (Order of the Minister of Health of the Republic of Kazakhstan from 21.12.2020, KR DSM-310/220; Order of the Minister of Health of the Republic of Kazakhstan from December 11, 2020 №KR DSM-248/2020) [6,7] and International Guidelines (The European Council Directive on the observance of ethical principles in work with laboratory animals (The European Council Directive (86/609/EEC)) and Directive 2010/63/EU of the European Parliament and Council of the European Union) [8,9].

Pathology induction (surgical intervention for skin wounds) was performed under anesthesia. Inhalation anesthesia with isoflurane (a drug approved for use in the territory of the Republic of Kazakhstan) was used as anesthesia, initially in the chamber, then with a mask. The rate of delivery was 4 liters/hour. Before applying full-skin wounds, the animal's hair was clipped with scissors in the area of the surgical field on the back between the shoulder blades. The hair remnants were removed with depilation cream, which was applied for 3 min. The operative field was then treated sequentially with 5% alcoholic iodine solution and 70º ethyl alcohol once. Using a sterile Derma-punch skin biopsy stylet with a diameter of 8 mm, 2 full-thickness skin wounds deep to the superficial fascia of the muscles were made in mice through the pulled back skin fold between the shoulder blades [3]. The excised skin was removed with forceps and scissors, then a 2-mm-thick silicone ring with an inner diameter of 10 mm was sutured (Vicryl 3-0 suture material). The presence of the wound did not cause discomfort to the animals, did not affect their motor activity and appetite.

For immunosuppression, hydrocortisone (125 mg) was administered at a dose of 25 mg/kg (intramuscularly) for 7 days (the 1st injection - 1 day before wounding) [10]. To obtain the desired concentration, hydrocortisone was diluted with sterile physiological solution.

Hydrocortisone belongs to the group of glucocorticosteroids (GCS), which are known to prevent the activation of phospholipase A2 by stimulating the formation of its inhibitor - lipocortin, disrupt the synthesis of prostaglandins and release of macrophage chemotactic factor, inhibit the activation of tissue kinins, reduce the migration of macrophages and lymphocytes into the focus of inflammation. The advantage of hydrocortisone in modeling purulent-inflammatory process is due to the fact that GCS suppress various stages of immunogenesis, but do not have mitostatic effect [11]. By affecting the functions of cells, which play an important role in wound healing, GCS cause a significant delay in the process of tissue repair. It is known that patients taking GCS or receiving other immunosuppressive therapy have a significant delay in skin wound healing [12].

After administration of hydrocortisone at the indicated dosage, two mice, i.e. 20%, died. The remaining 8 were single infected with a mixture of Staphylococcus aureus at varying concentrations from 10 in 6 to 10 in 12, and one overnight culture of Pseudomonas coli. The cultures were sequentially applied to the wounds with sterile cotton swabs

Pristane (2, 6, 10, 14-tetramethyl-pentadecane) in a dose of 0.5 ml (intraperitoneal) was used as the 2nd variant of immunosuppression once; surgical intervention was performed on the 7th day after administration. This drug was previously used in the literature to model systemic lupus erythematosus in animals.

After administration of Pristane preparation in the indicated dosage intraperitoneal, there was no death of animals during the observation period.

Two types of bacteria, S. Aureus and Pseudomonas aeruginosa, were used to infect wounds in order to produce a local inflammatory process. These strains was selected as the main nosocomial and out-of-hospital pathogens, also contributes significantly to the development of wound infections. S. Aureus is also a major pathogen commonly associated with diabetic foot osteomyelitis and can cause chronic and recurrent bone infections. Virulence of the pathogen and host immune factors may determine the occurrence and progression of S. Aureus infection [13].

Microbial cell concentration is expressed as the number of cells of the microorganisms to be determined (including non-viable and damaged cells) per unit volume of suspension. When determining the microbial cell concentration, the percentage of viable cells is determined by the number of live cells per unit volume of suspension (number of colony-forming units per ml - CFU/ml).

The microbial cell concentration counting procedure was performed manually. Visual counting was performed by determining the number of grown colonies after sowing microorganisms on LB nutrient media.

For cell titer determination, a commercial strain of Staphylococcus aureus ATCC 29213 was cultured overnight at 37o C, 150 rpm. In 5 ml LB broth.

1 ml of overnight culture was diluted in 9 ml of saline solution and seeded 100 µl each in a PE with LB. The colony-forming unit in 0.1 ml of the diluted culture was determined by the limit dilution method.

Cell titer was determined ∑=(x CFU /ml).

x CFU /ml - colony-forming unit count

Overnight culture of Staphylococcus aureus ATCC 29213 was grown in LB medium.

S. aureus cell titer reaches 11.63 *109 cells/0.1 ml, (2.32 *109 cells/0.05 ml) (Figure 1).

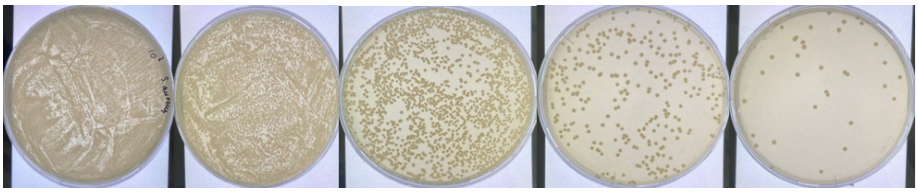

Figure 1 - Seeding of Staphylococcus aureus cultures on Petri dishes

An overnight culture of Pseudomonas aeruginosa ATCC was grown in LB medium.
*P. aeruginosa cell titer reaches 22.4*109 cells/0.1 ml, (1.12 * 109 cells/0.05 ml)*
CFU/mL - Colony forming unit in 0.1 ml of diluted culture

The wounds were infected once by different methods. A bacterial suspension of 106-10 CFU/mL (No 1, No 2, No 3, No 4, and No 5, respectively) in an amount of 0.1 ml of S. aureus and an overnight culture of P. Aeruginosa per wound was applied to the wound bed area. The 2nd method - application of suspension of overnight bacterial culture of S. aureus and P. Aeruginosa ("N.c." - overnight culture). After introducing microorganisms, the wound surface was isolated from the external environment with sterile transparent film for 2 days. The film was sewn to the surface of the silicone circle.

The wounds were photographed daily throughout the experiment. The obtained images were transferred to a computer, calibrated and the area of purulent plaque was measured using Scion Image program (NIH, USA).

After that, the plaque area on the 3rd day was calculated. The results were evaluated in the form of criteria - 0- absence of purulent plaque, 1- scanty plaque (up to 50% of the total wound area), 2- abundant purulent discharge (more than 50% of the total wound area). The number of microorganisms in the infected wounds was determined by sieving on the 3rd and 5th days of the experiment after wound infection. For this purpose, wound secretions were taken daily with a sterile swab and distributed on the surface of a dense nutrient medium (Mueller-Hinton agar). The cultures were placed in a thermostat and incubated for 24 h at 37ºC. The results of sowing, i.e. the number of colony-forming units, were counted and expressed in CFU/cm2.

Each animal was weighed before the start of the experiment and on subsequent days.

Statistical processing of the obtained data was performed using the program Statistic 19.

Results

After wound infection on the third and fifth day, the wound contents were taken for bacteriological sowing, as well as photo fixation and assessment of the presence or absence of purulent discharge from the bottom of the wound. The dynamics of the course of purulent-inflammatory process in the experiment was assessed by the appearance of plaque and the number of colony-forming units of microorganisms in the wound (Figure 2, Table1).

Fixation of the wound edges with a silicone ring made it possible to prevent premature tightening of the wound edges, which made it possible to "standardize" the wound area, regardless of the individual characteristics of the healing process in the experimental animal, as well as to evaluate the effect of the study drug. We chose a ring diameter of 10 mm (1 cm), which exceeds the wound diameter by 2 mm, to allow easy visual assessment of the appearance of purulent plaque (Figure 3-4).

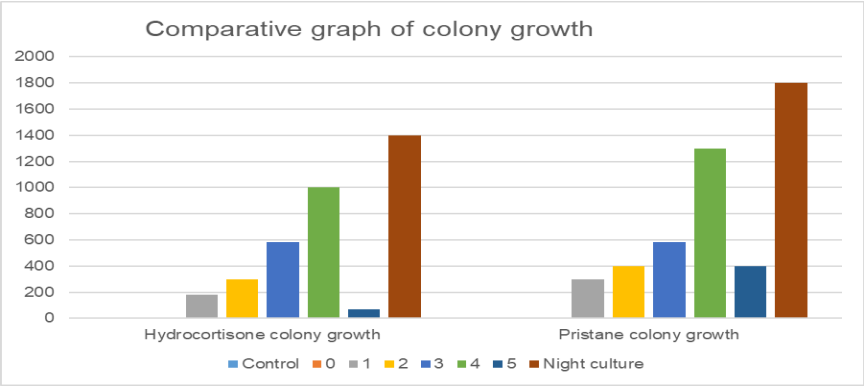

Figure 2 - Number of colony-forming units on the third day of the experiment

Table 1 - Colony forming unit count on the third day of the experiment

Samples	Control	0	1	2	3	4	5	N.c.
Hydrocortisone colony growth	0	5	180	300	585	1000	970	1400
Pristane colony growth	0	7	300	400	586	1300	1400	1800
Presence/absence of purulent plaque Hydrocortisone	0	0	0	0	1	1	2	2
Presence/absence of purulent plaque Pristane	0	0	0	1	1	2	2	2

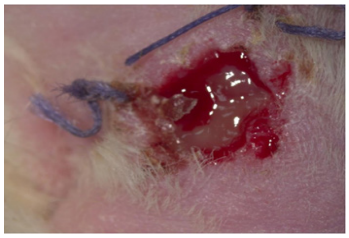

Figure 3 - Presence of purulent plaque on the third day. Immunosuppression with Hydrocortisone

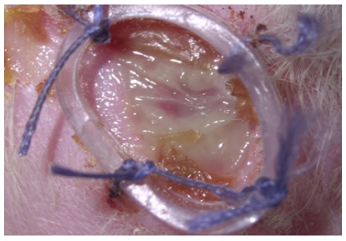

Figure 4 - Presence of purulent plaque on the third day. Immunosuppression with Pristane

According to the results of wound infection on the third day, purulent plaque was found in the groups of mice in which immunosuppression had been previously performed, while in the control group the wound had almost completely healed and was completely crusted (Figure 5).

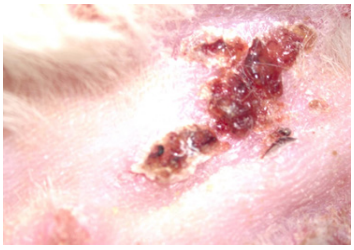

Figure 5 - Control group, without immunosuppression

As can be seen from the graph (Figure 6,7) and table (Table 2), the presence of purulent plaque is detected from the second titer in Pristane immunosuppression, and from the third titer in mice after administration of hydrocortisone. A direct correlation, i.e. dependence of the results of bacteriologic seeding on the initial titer of canthamination, excluding titer #5, was also found, which is probably related to the insufficient quality of this particular dilution, since lower figures were obtained in all 4 mice (Figure 8,9).

Moreover, in mice after induction of immunity by Pristane, the number of colony-forming units in all cultures exceeded the results after induction by Hydrocortisone.

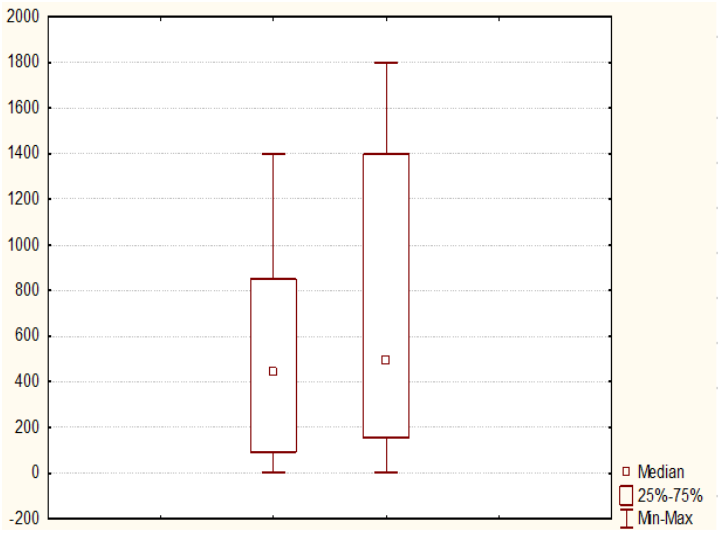

Figure 6 - Number of colony-forming units on the third day of the experiment. Comparative characterization of the number of CFU (colony-forming units) in bacteriological cultures from wounds of mice under immunosuppression with Hydrocortisone and Pristane

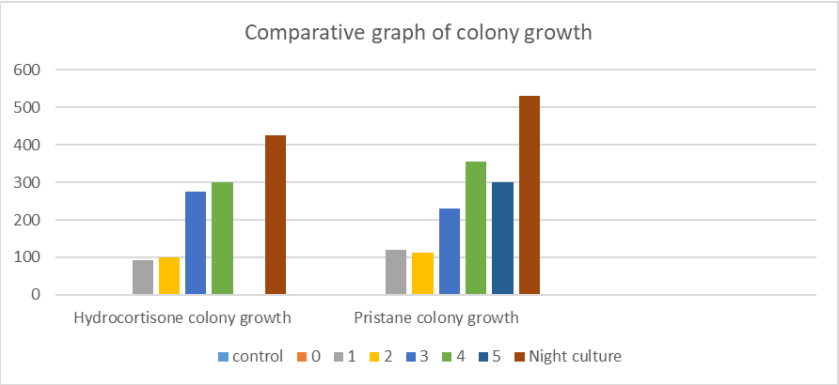

Figure 7 - Number of colony-forming units on the fifth day of the experiment

Table 2 - Number of colony forming unit on the fifth day of the experiment

Samples	Control	0	1	2	3	4	5	N.c.
Hydrocortisone colony growth	0	0	93	100	276	300	0	425
Pristane colony growth	0	0	120	112	230	354	300	530
Presence/absence of purulent plaque Hydrocortisone	0	0	0	0	0	1	1	2
Presence/absence of purulent plaque Pristane	0	0	0	0	1	1	2	2

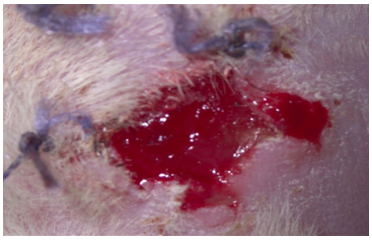

Figure 8 - Presence of purulent plaque on the fifth day. Immunosuppression with Hydrocortisone

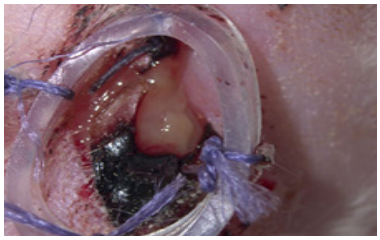

Figure 9 - Presence of purulent plaque on the fifth day. Immunosuppression with Pristane

According to the results of wound fixation on the fifth day purulent plaque was found in the groups of mice in which immunosuppression with the drug Prestan from the 4th titer was previously performed, while in Hydrocortisone - 5 and overnight culture also, in the control group the wound was completely healed. An area of necrosis of the wound edge was found in the mouse from the Prestan group (Figure 10).

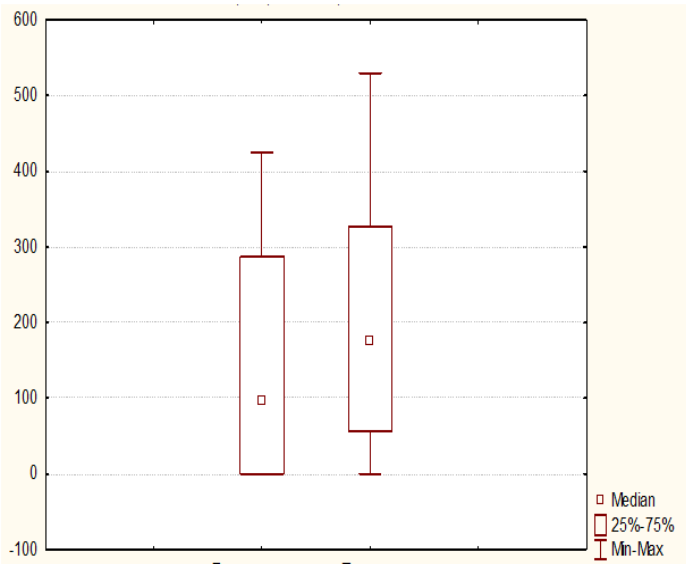

Figure 10 - Number of colony-forming units on the fifth day of the experiment. Comparative characterization of the number of colony forming unit (colony-forming units) in bacteriological cultures from wounds of mice under immunosuppression with Hydrocortisone and Pristane

Discussion

Modeling of the wound process, including wound infection, is a rather difficult task [14]. Currently, there are several ways of modeling a complicated purulent wound process in animals, most of them using immunosuppression. Reduction of immunity is done both locally, e.g. using physical methods (sponges, gauze swabs in wounds) [15], and generalized with the administration of drugs. Hormonal drugs are often used as immunosuppression, but modeling of the process is complicated by the need for daily administration of the drug for several days, as well as a certain number of lethal outcomes in experimental animals [16].

This study evaluated the adequate reproduction of complicated wounds in a preclinical study in mice using hydrocortisone and the chemical compound 2, 6, 10, 14-tetramethyl-pentadecane, which up to this point has been used exclusively for modeling autoimmune processes [17].

Administration of 2, 6, 10, 14-tetramethylpentadecane in the dosages indicated earlier [18] allowed to achieve reproduction of the infected wound, with bacterial contamination exceeding that of Hydrocortisone. The advantage was also the fact that 2, 6, 10, 14-tetramethylpentadecane was administered once compared to the hormonal preparation, which had to be administered daily for a week.

The limitation of this study is the use of only one drug - Hydrocortisone as a comparison group, nevertheless, it was taken into account that it is one of the most common and available drugs used for modeling the wound process in experimental animals.

Conclusions

Having analyzed the obtained results we can say that the use of Pristan as immunosuppressive therapy is possible and quite justified, along with the currently existing methods. Thus, its use in comparison with Hydrocortisone significantly simplifies the procedure of immune response induction. Instead of daily injections, the use of 2, 6, 10, 14-tetramethylpentadecane requires only a single manipulation, while the quality of immune suppression is not lost. In our work, we were able to try to standardize the size of the wound, which made the results more reliable.

This study may be useful in modeling the complicated wound process using immunosuppression with drugs during preclinical studies.

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Conflict of interest The authors declare that there is no conflict of interest in this paper.

Contribution of the authors. A.A.M - conceptualization, conducting the experiment itself, collecting data, writing the draft version, statistical processing of data; A.J.S. - building preclinical research methodology, guiding the experiment, formal analysis, editing; C.G.C. - participating in an experiment, assisting with a trial, collecting data, structuring, editing; M.A.E. - Participate in an experiment, assist in the conduct of the trial, collect data, edit, and preliminarily evaluate the results obtained; N.A.C. - participating in the experiment, assisting with the trial, data collection, structuring, editing, statistical processing of data.

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Ob utverzhdenii pravil provedenija klinicheskih issledovanij lekarstvennyh sredstv i medicinskih izdelij dlja diagnostiki vne zhivogo organizma (in vitro) i trebovanija k klinicheskim bazam i okazanija gosudarstvennoj uslugi "Vydacha razreshenija na provedenie klinicheskogo issledovanija i (ili) ispytanija farmakologicheskih i lekarstvennyh sredstv, medicinskih izdelij" (On the approval of the rules for conducting clinical research of medicinal products and medical products for in vitro diagnostics and requirements for clinical bases and provision of state services) [in Russian]. Prikaz Ministra zdravoohranenija Respubliki Kazahstan; ot 11 dekabrja 2020 goda, № KR DSM-248/2020. Rezhim dostupa: https://adilet.zan.kz/rus/docs/V2000021772/info

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Гидрокортизон және 2,6,10,14-тетраметилпентадеканды қолдану арқылы иммуносупрессия фонында тышқандардағы іріңді-қабыну процесін модельдеу. In vivo зерттеу

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Түйіндіме

Әртүрлі этиологиялы жаралардың іріңді асқынулары заманауи медицинаның өзекті мәселелерінің бірі болып табылады. Антибиотиктерге төзімділік қазіргі уақытта барған сайын жаңа және жетілдірілген дәрілердің дамуына немесе қолданыстағылардың формалары мен жеткізу әдістерін жаңартуға серпін беруде.

Зерттеудің мақсаты тышқандардағы іріңді жара үлгісін көбейту және гидрокортизон мен 2, 6, 10, 14-тетраметилпентадекан қолдану арқылы иммуносупрессия әдістерін салыстыру болды.

Әдістері. Тышқандар мен егеуқұйрықтардағы тері жараларындағы іріңді-қабыну процесінің дамуын модельдеудің бірнеше нұсқалары орындалды. Тышқандарға тәжірибе жүргізген кезде үш топқа бөлінді: 1. Гидрокортизонды иммуносупрессия ретінде қолдану (25 мг/кг 7 күн бойы) Жаралар препаратты енгізудің екінші күні қолданылды. 14 тұлғаның нәтижелері бойынша. 2. Пристане препаратын иммуносупрессия ретінде қолдану (1 адамға 500 мкл іші арқылы, 1 рет). Жаралар 7-ші күні жағылды. 14 тұлғаның нәтижелері бойынша. 3. Бақылау тобында 14 тышқан қолданылды – иммуносупрессиясыз. Содан кейін жараны жұқтыратын микроорганизмдер ретінде бактериялардың екі түрі тексерілді: қалыпты тері микробиотасының өкілі алтын стафилококк және ауруханаішілік инфекциялардың қоздырғыштары псевдомоназдардың ең көп таралған түрі ретінде Pseudomonas aeruginosa. Жара инфекциясы жоғарыда аталған 2 бактериалды дақылдың аралас суспензиясын қолдану арқылы жүргізілді.

Нәтижесі. Іріңді жараларды алудың ең оңтайлы моделі анықталды, атап айтқанда 2, 6, 10, 14-tetramethylpentadecane препаратымен иммуносупрессияны қолдану нұсқасы. Бұл препаратты қолдану иммуносупрессивті инъекциялардың санын азайтуға және жараның бетінде тығыз биопленканы қалыптастыруға мүмкіндік берді.

Қорытынды. Тышқандардағы іріңді жараны модельдеу 2, 6, 10, 14-tetramethylpentadecane және гидрокортизон препараттары болуы мүмкін иммуносупрессия фонында ғана мүмкін деген қорытындыға келді.

Түйін сөздер: іріңді жара моделі, иммуносупрессия, жануарлардағы иммуносупрессияға арналған препараттар.

Моделирование гнойно-воспалительного процесса у мышей на фоне иммуносупрессии с использованием гидрокортизона и 2,6,10,14-тетраметилпентадекана. Исследование in vivo.

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Резюме

Гнойные осложнения ран различной этиологии является одной из актуальных проблем современной медицины. Антибиотикорезистентность в настоящее время дает толчок развития все более новых и совершенных препаратов, либо модернизации форм и методов доставки уже существующих. Устойчивый иммунитет животных, а именно мышей и крыс, создает препятствия в проведении доклинических исследований данных препаратов, ввиду сложности моделирования гнойно-воспалительного процесса. Рассмотренные в различных источниках модели воспроизводства раневой инфекции кожного покрова животных даже с применением иммуносупрессорной терапии к сожалению, не всегда удается применить на практике.

Целью исследования являлось воспроизведение модели гнойной раны у мышей, и сравнение методов иммуносупрессии с применением гидрокортизона и препарата 2,6,10,14-tetramethyl-pentadecane.

Методы. Выполнены несколько вариантов моделирования развития гнойно-воспалительного процесса в кожных ранах у мышей и крыс. При проведении эксперимента на мышах было выделено три группы: 1. С применением в качестве иммуносупрессии гидрокортизона (из расчета 25 мг/кг в течении 7 дней) Раны наносились на второй день введения препарата. На основании результатов 14 особей. 2. С применением в качестве иммуносупрессии препарата Пристан (из расчета 500 мкл внутрибрюшинно на 1 особь 1 раз). Раны наносились на 7 день. На основании результатов 14 особей. 3. 14 мышей были использованы в группе контроля – без иммуносупрессии. Затем в качестве инфицирующих раны микроорганизмов апробировали бактерии 2 видов: Staphylococcus aureus – представитель нормальной микробиоты кожи и Pseudomonas aeruginosa – как наиболее распространенный вида псевдомонад – возбудителей внутрибольничных инфекций. Инфицирование ран проводили, используя смешанную суспензию 2 указанных выше бактериальных культур.

Результаты. Мы определили наиболее оптимальную модель получения гнойных ран, а именно вариант с применением иммуносупрессии препаратом 2, 6, 10, 14-tetramethyl-pentadecane. Применение данного препарата позволило сократить количество введений иммуносупрессора, получить более плотную биопленку на поверхности раны.

Выводы. Моделирование гнойной раны у мышей возможно только на фоне иммуносупрессии, в качестве которой могут применятся препараты 2, 6, 10, 14-tetramethyl-pentadecane и гидрокортизон.

Ключевые слова: модель гнойной раны, иммуносупрессия, препараты для иммуносупрессии у животных.