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ANTIBACTERIAL AND IMMUNOTROPIC PROPERTIES OF ISOLIQUIRITIGENIN IN GENERALIZED STAPHYLOCOCCAL INFECTION IN MICE

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The article is devoted to the study of the effects of isoliquiritigenin in generalized bacterial infections.
The aim is to study antibacterial and immunotropic mechanisms and effects of isoliquiritigenin in generalized staphylococcal infections in a mouse model.
Materials and methods. To assess the survival rate of Balb/C mice, a generalized infection model caused by *Staphylococcus aureus J49 ATCC 25923* with Kaplan-Meier curves was used. The degree of bacteremia during the development of infection was determined by the method of sector crops. The minimum inhibitory concentration of isoliquiritigenin against *Staphylococcus aureus J49 ATCC 25923* was determined by serial dilutions methods. To study an antibiofilm activity, the MTT test and atomic force microscopy were used. Immunotropic effects were studied by assessing peptone-induced migration of phagocytes into the abdominal cavity, proliferation of mitogen-activated lymphocytes in the MTT test and their cytokine secretion using the MILLIPLEX MAP kit on a Magpix multiplex analyzer.
Results. It has been established that a preliminary intraperitoneal administration of isoliquiritigenin (30 mg/kg) increases the survival rate of Balb/C mice in case of generalized staphylococcal infections. Isoliquiritigenin has antibacterial (MOC = 64 μg/ml) and antibiofilm (4–32 μg/ml) activities against *S. aureus J49 ATCC 25923,* does not inhibit the migration of phagocytes in the abdominal cavity, dose-dependently inhibits the proliferation and secretion of cytokines by mitogen-activated T-lymphocytes and modulates the production of cytokines (IL-2, IL-12p70, IFNg, TNFα, IL-6, IL-22, IL-23, IL-17A, IL-17F, IL-17E/IL-25, GM-CSF, MIP – 3a/CCL20, IL-10) by the cells of inguinal lymph nodes and splenocytes in the early stages of generalized staphylococcal infections.
Conclusion. A preliminary administration of isoliquiritigenin increases the survival rate of mice with generalized staphylococcal infections, which may be associated with both antimicrobial (antistaphylococcal, antibiofilm) and immunotropic mechanisms. The obtained data on the pharmacodynamics of isoliquiritigenin deserve attention from the point of view of the prospects of the new drugs creation that reduce mortality in staphylococcal sepsis.
Keywords: antimicrobial activity, biofilms, isoliquiritigenin, immunity, Balb/C mice, *S. aureus*
Abbreviations: MHB – Mueller-Hinton Broth; DMSO – dimethyl sulfoxide; ISL – isoliquiritigenin; SI – stimulation index; CFU – colony forming unit; ConA – concanavalin A; MIC – minimal inhibitory concentration; PBS – phosphate buffered saline; GM-CSF – colony stimulating factor 2 (granulocyte-macrophage); IFNg – interferon-gamma; IL – interleukin; MIP-3a/CCL20 – Macrophage Inflammatory Protein-3/Chemokine (C-C motif) ligand 20; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; OD – optical density; S. aureus – *Staphylococcus aureus;* SD – standard deviation; Th – T-helper cell; TNFα – tumor necrosis factor alpha.

АНТИБАКТЕРИАЛЬНЫЕ И ИММУНОТРОПНЫЕ СВОЙСТВА ИЗОЛИКВИРИТИГЕНИНА ПРИ ГЕНЕРАЛИЗОВАННОЙ СТАФИЛОКОККОВОЙ ИНФЕКЦИИ У МЫШЕЙ

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RESEARCH ARTICLE

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Статья посвящена изучению эффектов изоликвиритигенина при генерализованной бактериальной инфекции.
Цель: изучение антибактериальных и иммунотропных механизмов и эффектов изоликвиритигенина при генерализованной стафилококковой инфекции в мышиной модели.
Материалы и методы. Для оценки выживаемости мышей линии Balb/C использовали модель генерализованной инфекции, вызванной *Staphylococcus aureus J49 ATCC 25923* с построением кривых Каплан-Мейера. Степень бактериемии при развитии инфекции определяли методом секторных посевов. Минимальную подавляющую концентрацию изоликвиритигенина в отношении *Staphylococcus aureus J49 ATCC 25923* определяли методом серийных разведений. Для исследования антибиопленочной активности использовали МТТ-тест и атомно-силовую микроскопию. Иммунотропные эффекты изучали, оценивая пептон-индуцированную миграцию фагоцитов в брюшную полость, пролиферацию митоген-активированных лимфоцитов в МТТ-тесте и секрецию ими цитокинов с помощью набора MILLIPLEX MAP на мультиплексном анализаторе Magpix.
Результаты. Установлено, что предварительное внутрибрюшинное введение изоликвиритигенина (30 мг/кг) увеличивает выживаемость мышей Balb/C при генерализованной стафилококковой инфекции. Изоликвиритигенин обладает антибактериальной (МПК = 64 мкг/мл) и антибиопленочной (4–32 мкг/мл) активностью в отношении *S. aureus J49 ATCC 25923,* не ингибирует миграцию фагоцитов брюшную полость, дозозависимо подавляет пролиферацию и секрецию цитокинов митоген-активированными Т-лимфоцитами и модулирует выработку цитокинов (IL-2, IL-12p70, IFNg, TNFα, IL-6, IL-22, IL-23, IL-17A, IL-17F, IL-17E/IL- 25, GM-CSF, MIP-3a/CCL20, IL-10) клетками паховых лимфатических узлов и спленоцитов на ранних стадиях генерализованной стафилококковой инфекции.
Заключение. Предварительное введение изоликвиритигенина повышает выживаемость мышей при генерализованной стафилококковой инфекции, что может быть связано как с антимикробными (антистафилококковым, антибиопленочным действием), так и иммунотропными механизмами. Полученные данные о фармакодинамике изоликвиритигенина заслуживают внимания с точки зрения перспективы создания новых лекарственных препаратов, снижающих летальность при стафилококковом сепсисе.
Ключевые слова: антимикробная активность, биопленки, изоликвиритигенин, иммунитет, мыши, *S. aureus*
Сокращения: БМХ – бульон Мюллера-Хинтона; ДМСО – диметилсульфоксид; ИЛГ – изоликвиритигенин; ИС – индекс стимуляции; КОЕ – колониеобразующие единицы; КонА – конканавалин А; МПК – минимальная подавляющая концентрация; ФСБ – фосфатно-солевой буфер; GM-CSF – гранулоцитарно-макрофагальный колониестимулирующий фактор; IFNg – интерферон-гамма; IL – интерлейкин; MIP-3a/CCL20 – макрофагальный белок воспаления-3а/хемокиновый лиганд (CC) 20; МТТ – 3-(4,5-диметилтиазол-2-ил)-2,5-дифенил-тетразолиум бромид; OD – оптическая плотность; *S. aureus* – *Staphylococcus aureus*; SD – стандартное отклонение; Th – Т-хелперы; TNFα – фактор невроза опухоли альфа.

INTRODUCTION

Staphylococcus aureus (S. aureus) is a pathogen that causes severe generalized infections in humans. Among the infections caused by gram-positive bacteria, *S. aureus* infection is characterized by high mortality due to the development of sepsis and septic shock [1]. A septic process is known to be accompanied by a "cytokine storm" leading to a multiple organ failure. At the same time, an early prescription of antibacterial drugs is not always effective due to the development of the uncontrolled systemic inflammation, as well as the resistance of *S. aureus* to antibiotics [2]. Currently, low doses of corticosteroids which have undesirable immunosuppressive effects, are recommended to reduce mortality in septic shock in this situation [3]. Thus, many aspects of the treatment of sepsis remain controversial and require an in-depth fundamental study.

In case of massive generalization of infections, the reaction of the immune system is known to take on the features of systemic inflammation with a multiple organ failure, the main pathogenetic factor of which is the production of pro-inflammatory cytokines that trigger the generation of free radicals [4]. *S. aureus* can produce a toxic shock syndrome toxin [5], which acts as a superantigen able of inducing cytokine release at low concentrations, triggering the development of a "cytokine storm".

Recent studies have shown that licorice root flavonoids increase the secretion of IL-17 by activated T cells *in vitro* [6], and also lead to a switch of the immune response with differentiation of IL-17-producing cells in a contact sensitivity model [7]. Moreover, in the model of generalized staphylococcal infections, a preliminary administration of the sum of licorice flavonoids increased the survival rate of the laboratory animals [8].

Isoliquiritigenin (ISL) is one of the main flavonoids of licorice roots, which has various types of pharmacological activity: antitumor [9], antimicrobial [6], as well as anti-inflammatory and immunomodulatory [9–11]. These properties make it relevant for studying licorice roots as an agent in generalized infectious and inflammatory processes. In this study, an attempt was made to experimentally substantiate the use of chalcone isoliquiritigenin (ISL) in generalized infections (in the mice), caused by *S. aureus.*

THE AIM of this work was to study antibacterial and immune mechanisms of ISL in sepsis in mice, caused by an intraperitoneal administration of *S. aureus J49 ATCC25923* strain.

MATERIALS AND METHODS

Bacterial strain and conditions for its cultivation

S. aureus J49 ATCC25923 strain, obtained from the Federal State Budgetary Institution "Scientific Center for Expertise of Medicinal Products" of the Ministry of Health of Russia (Moscow, Russia), was grown in Mueller-Hinton broth (MHB, Medica plus LLS, Russia) at 37 °C in glass vials with aeration. For experimental purposes, a medium log phase bacterial culture was used, which had been cultured in 96-well flat-bottomed plates. (Corning Costar, USA). The calculation of colony forming units (CFUs) was carried out by measuring the optical

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density (OD) of the bacterial suspension at 630 nm using a microplate photometer (ImmunoChem 2100, USA) based on the following ratio: 1 optical unit OD630 = 8.5×10^8 CFUs/ml.

Isolation of mice's mononuclear cells and their cultivation conditions

The isolation of mononuclear cells from inguinal lymph nodes or mice's spleen, was performed by gentle homogenization in RPMI-1640 (Thermo Fisher Scientific, USA) with osmotic lysis of erythrocytes in a 0.15 M ammonium chloride solution. The isolated lymphoid cells were cultured at 37 °C, 100% humidity, and 5% CO_2 in RPMI-1640 supplemented with 10% inactivated fetal calf serum (Thermo Fisher Scientific, USA), penicillin (100 U/ml), streptomycin (100 µg/ml) ("a complete medium") in 96-well round-bottom cell culture plates (Corning Costar, USA). Concanavalin A (ConA, PanEco LLC, Russia) at the final concentration of 15 µg/ml was used to activate T cells.

Test agent

ISL (98% purity, Xi'An Yiyang Bio-Tech Co., China) was dissolved in dimethyl sulfoxide (DMSO, Panreac, Spain). In the experiments *in vitro*, ISL was tested in the concentration range so that the final concentration of DMSO in the test samples did not exceed 1%. In the control samples, the corresponding volumes of DMSO were added instead of ISL. In the experiments on the animals, ISL was injected intraperitoneally three times with an interval of 4 h in a single dose of 10 mg/kg in 0.5 ml of phosphate-buffered saline at pH 7.4 (PBS, PanEco LLC, Russia).

Experimental animals

Balb/C mice (males, 20–22 g, 6–8 weeks old) were obtained from the Research and Production Enterprise "Nursery for Laboratory Animals" of the Institute of Biology, the Russian Academy of Sciences (Pushchino, Russia). The animals were cared for and handled in accordance with the ARRIVE principles [12]. The animals were kept with a free access to water and food. For the experiments, the mice were randomly assigned to groups of 8 animals. Withdrawal from the experiment was carried out by decapitation or cervical dislocation.

When performing the experiments, the provisions of the Helsinki Declaration (Brazil, 2013) were observed, the protocol of these experiments was approved by the ethical committee of Chuvash State University n. a. I.N. Ulyanov" (Protocol No 20-04 dated 17 April, 2020).

Determination of antimicrobial activity

The antimicrobial activity was determined by the dilutions method in Mueller-Hinton broth in 96-well flat-bottomed plates [13]. The serial two-fold dilutions of ISL (with the final concentration range of 0.1–128 µg/ml) were added to the bacterial suspension of *S. aureus* ($5x10^5$ CFUs/ml) and incubated at 37 °C for 24 hours. The minimum inhibitory concentration (MIC) of ISL was considered the lowest concentration with no visible bacterial growth after the incubation time.

Assessment of the dynamics of bacterial growth

To assess a bacterial growth, the method described by Wang [14], was used with minor modifications. ISL was added to the bacterial suspension (5×10^5 CFUs/ml) so that the final concentrations of ISL in the samples were 1/8 MPK, 1/4 MPK, 1/2 MPK, MPK. To assess bacterial growth in the samples, OD was measured after 4, 8, 12, 24 hours at 630 nm using a microplate photometer.

MTT test for bacterial biofilm formation

To study the formation of bacterial biofilms, the method described by Grela [15] was used. Bacteria (5×10^5 CFUs/ml) were inoculated into 96-well flat-bottomed cell culture plates and cultured for 24 hours. 2 hours before the end of the cultivation, then the bacterial suspension was removed, the wells were washed three times with PBS and a 1% solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-dislenyltetrazolium bromide (MTT, eBioscience, USA) was added into PBS and incubated for 2 hours at 37 °C. After that, to dissolve the formazan particles, the MTT solution was replaced with DMSO and incubated for 15 min at 20 °C, then the OD was measured at 492 nm using a microplate photometer.

Model of Systemic S. aureus Infection in Mice

S. aureus suspension was injected in PBS intraperitoneally: 5×10^8 CFUs/mouse, 1.5×10^9 CFUs/mouse. The infecting day was considered zero. The survival was assessed every 6 hr on the first day, then every day for 24 days. The experimental animals were injected with ISL before infecting (the total dose was 30 mg/kg, intraperitoneally, three times every 4 hours). The control animals were administrated with 5% DMSO.

The mice infected with a sublethal dose of *S. aureus* (5×10^8 CFUs/mouse) were withdrawn from the experiment every day for 7 days, to collect blood from large vessels (determination of bacteremia) and determine the excretions of the spleen and inguinal lymph nodes.

Determination of bacteremia

Bacteremia was determined by methods of sector inoculations методом секторных посевов on Petri dishes [16] with blood agar. The cups were incubated at 37 °C for 24 hours, then CFUs per 1 ml were calculated.

Peptone-induced phagocyte migration

The migration of phagocytes into the abdominal cavity was assessed according to the method proposed by Miyazaki [17], with minor changes. For this, group 1 of the negative control was injected three times with sterile PBS (0.5 ml, intraperitoneally); group 2 was injected with a sterile solution of peptone in PBS (3% –3 ml, intraperitoneally); group 3 was injected three times

with DMSO (5% – 0.5 ml, intraperitoneally), then with a sterile solution of peptone in PBS (3% – 3 ml, intraperitoneally). The mice of group 4 were injected with ISL three times, then with a sterile peptone solution in PBS (3% – 3 ml, intraperitoneally). After 24 hours and 72 hours, the animals withdrawn from the experiment, were injected intraperitoneally sequentially with 20 ml of PBS. After the palpation of the abdomen, the resulting washings were taken into plastic tubes and centrifuged. The number of cells was counted by a light microscopy using a Goryaev camera. The stimulation index (SI) was calculated using the following formula: SI = A/B, where A is the number of cells in the groups receiving peptone, B is the number of cells in the negative control group.

Determination of cytokines

On days 4 and 5 after infecting, the cells of the spleen and inguinal lymph nodes (5×10^6 cells/ml) of infected (5×10^8 CFUs/mouse) or intact mice were cultured for 24 or 48 hours at 37 °C in 100% humidity and 5% CO_2 in "a complete medium" with the addition of ConA. The supernatants had been collected and stored at – 70 °C till the analysis with a reagent kit for the determination of Mouse Th17 cytokines – MILLIPLEX MAP, Mouse Th17 MAGNETIC BEAD PANEL KIT 96-Well Plate Assay (USA); then they were analyzed using a multiplex analyzer (Magpix, USA) to determine the concentration of cytokines IL-2, IL-12p70, interferon gamma (IFNg), a necrosis factor of alpha tumor (TNFα), IL-6, IL-22, IL-23, IL-17A, IL-17F, IL- 17E/IL-25, a granulocyte macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein-3 (MIP-3a/CCL20), IL-10.

Statistical analysis

All the experiments have been performed in at least three series. The data obtained during them, were statistically processed using the GraphPadPrism 8.4.0 software. To assess the dynamics of the mice's deaths, Kaplan-Meier curves were constructed. The results obtained, complied with the law of normal distribution, were processed by the methods of variation statistics, and were presented as the arithmetic mean (M) ± standard error of the mean (SEM). The significance of differences between the groups in the experiments was determined by the Student's test. The differences were considered significant at $p<0.05$, where p is the level of significance.

RESULTS

Effect of ISL on the survival of Balb/C mice infected with *S. aureus J49 ATCC 25923*

Infecting with $5x10^8$ CFUs/mouse did not lead to deaths in the control group, although the animals showed such symptoms as a decreased activity and appetite, tousled coats, diarrhea. The survival rate of the healthy animals, which had been injected three times intraperitoneally with 5% DMSO, was 100%.

Intraperitoneal injection of 1.5×10^9 CFUs/mouse caused the deaths of 100% of the animals in the control group within 48–72 hours. A preliminary administration of ISL significantly reduced the mortality: 62.5±12.5% of mice died after 2 days, 12.5±6.3% of mice survived by day 24 (Fig. 1).

Autopsy made it possible to establish that 48–72 after infecting the mice treated with ISL, there was a less pronounced injection of mesenteric vessels and intestinal distention. In the ISL group, 14 days later, a pronounced adhesive process was observed, and 24 days later, retroperitoneal abscesses with dense capsules and adhesions were notified.

Antimicrobial activity of ISL

To determine the MIC against *S. aureus J49 ATCC 25923,* ISL was tested in the concentration range of 0.1 – 128 μg/ml. After 24 hours, the samples with ISL at the concentrations of 128 μg/ml and 64 μg/ml were completely transparent, like the negative control (sterile MHB). The bacterial suspension with ISL at the concentration of 32 μg/ml was opalescent. In the rest of the samples with ISL (0.1–16 μg/ml), an intensive bacterial growth was observed, as in the positive control (bacterial suspension without ISL). Thus, the MIC of ISL against *S. aureus J49 ATCC 25923* was 64 μg/ml.

As Fig. 2 shows, ISL dose-dependently suppressed the growth of the studied strain of *S. aureus* at the concentrations of MIC – MIC/8. In the first 4 hours of the observations, the optical density of the samples did not differ significantly, while after 8 h the differences in OD630 between the positive control and the samples with ISL increased. By the end of the incubation (24 hours), OD630 for ISL in MIC was 0.1±0.0 ($p<0.05$), MIC/2 was 0.3±0.1 ($p<0.05$), MIC/4 was 0.4±0.1 ($p<0.05$), MIC/8 was 0.5±0.3 ($p<0.05$).

Effect of ISL on biofilm formation of *S. aureus J49 ATCC 25923*

Using the MTT test, it was found out that ISL dose-dependently reduces the ability of *S. aureus J49 ATCC 25923* to adhere to plastic (Fig. 3). The removal of the bacterial suspension with repeated washings of the wells of the culture plate after 24 hours of incubation showed that the optical density of washings from the plastic surface in the control samples was significantly higher than in the wells with ISL: 0.8±0.1 *vs.* 0.4±0.0 (MIC, $p<0.05$), 0.8±0.1 *vs*. 0.5±0.1 (MIC/2, $p<0.05$), 0.8±0.1 *vs.* 0.5±0.2 (MIC/4, $p<0.05$), 0.8±0.1 *vs.* 0.5±0.1 (MIC/8, $p<0.05$). The OD492 value in the wells with ISL MIC/16 also tended to decrease: 0.7±0.1 ($p>0.05$).

Using the atomic force microscopy, it was found out that the number of bacteria in the field of view in the samples with MIC ISL (8.0±2.0 bacteria in the field of view, 300×, $p<0.05$), MIC/2 (30.0±7, 0 bacteria in the field of view, 300×, $p<0.05$) was fewer than in the control samples with massive bacterial conglomerates (83.0±13.0 bacteria in the field of view, 300×) (Fig. 3).

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Effect of ISL on peptone-induced phagocyte migration in mice

The migration of phagocytes into the abdominal cavity was assessed by calculating the stimulation index (SI) – the number of cells stimulated by intraperitoneal injection of peptone relative to PBS. The SI in mice treated with ISL, and the control animals stimulated with peptone, did not differ from each other significantly. After 24 hours, the SI in the control group was 2.4±0.1 *vs*. 2.0±0.1 of the group administrated with ISL; after 72, the SI values were characterized by values 1.6±0.1 (control group) compared with 1.8±0.1 (the group administrated with ISL).

Dynamics of splenocytes and cells of inguinal lymph nodes in generalized staphylococcal infection in Balb/C mice

The number of the cells was counted every day after the intraperitoneal infection with a sublethal concentration of bacteria (5×10^8 CFUs/mouse) for 2 weeks. The dynamics of the number of the cells is shown in Fig. 5. On the 1st day after infecting, the number of the cells in the inguinal lymph nodes in the mice treated with ISL ($0.4\pm0.2\times10^6$ cells/mouse) and control mice ($0.9\pm0.4\times10^6$ cells/mouse), decreased compared with intact mice ($2.3\pm1.1\times10^6$ cells/mouse), and only after the 3rd day it gradually increased in both groups, reaching maximum values on the 7th day ($9.3\pm0.5\times10^6$ cells/mouse) or on the 10th day 10 ($10.6\pm0.5\times10^6$ cells/mouse, the ISL group). After reaching a peak on days 9–10 of the development of the infection, the number of lymph node cells gradually decreased, reaching their normal values (in intact animals) in both groups by the 16th day.

In the first days after infecting, the number of splenocytes in both groups was comparable and fewer than in the uninfected mice ($363.0\pm125.4\times10^6$ cells/mouse), up to the 6th day. From 3 to 9 days, the number of splenocytes gradually increased in both groups. The number of splenocytes in the mice treated with ISL reached a maximum by day 10 ($3328.0\pm166.4\times10^6$ cells/mouse). The control group had similar dynamics with a maximum on day 10, but with a lower peak value ($1488.0\pm74.4\times10^6$ cells/mouse).

Effect of ISL on proliferation of splenocytes and their cytokine secretion in vitro

According to the results of the MTT test with the use of the T-cell mitogen ConA, it was found out that ISL at the concentrations of 4–64 μg/ml dose-dependently suppresses the proliferation of activated lymphocytes. Thus, ISL at the concentrations of 16–64 μg/ml almost completely suppressed the cell proliferation. In the presence of 8 μg/ml of ISL, the cell viability decreased more than twice (50.0±7.5%, $p<0.05$) in comparison with the control and had a tendency to decrease upon the exposure to ISL at the concentration of 4 μg/ml (88.0±22.0%).

In the samples with ISL, even at the concentration of 4 μg/ml (Table 1), after 24-48 hours of incubation, the level of almost all the studied cytokines was lower than in the control samples.

Effect of ISL on bacteremia

To detect bacteremia, the blood of the infected mice (5×10^8 CFUs/mouse) was inoculated on the blood agar. A significant bacterial growth was observed one day later after infecting the control mice (10^5 CFUs/ml) compared with the mice treated with ISL (no growth was observed). On days 4-5 of the infection in the group administrated with ISL, there was no marked bacterial growth in the blood samples ($<10^3$ CFUs/ml). In the other samples of the control and experimental groups, no growth was observed, either.

Effect of ISL on cytokine production by cells of inguinal lymph nodes in generalized staphylococcal infections in Balb/C mice

The secretion of cytokines by the cells of the inguinal lymph nodes was determined on the 4th and 5th days after infecting (5×10^8 CFUs/mouse), by incubating the cells for 24–48 hours *in vitro*. As Fig. 5 and 6 show, the production of many cytokines detected on the 4th day of the infection was higher compared to the 5th day after infecting.

By 48 h of the incubation, the levels of IL-2, IFNg, IL-6, GM-CSF and, in particular, IL-17A, had been characterized by rather high values in the infected control mice. The intraperitoneal administration of ISL prior to the infection in the mice, significantly reduced the production of such cytokines as IL-2 (5524.3±669.8 pg/ml *vs*. 1265.0±94.8 pg/ml, $p<0.05$), IFNg (3936.3±567.8 pg/ml *vs*. 587.6±20.9 pg/ml, $p<0.05$), IL-6 (4861.3±361.8 pg/ml *vs*. 412.3±11,8 pg/ml, $p<0.05$), GM-CSF (553.3±64.6 pg/ml *vs.* 80.3±6.3 pg/ml, $p<0.05$), and IL-17A (6804.0± 754.9 pg/ml *vs.* 1129.0±31.1 pg/ml, $p<0.05$).

Effect of ISL on cytokines produced by splenocytes during staphylococcal infection in Balb/C mice

The secretion of cytokines by splenocytes was determined on days 4 and 5 after infecting (5×10^8 CFUs/mouse) by incubating the cells for 24–48 *in vitro*. The administration of ISL to mice led to a gradual increase in the secretion of cytokines from 4 to 5 days after infecting. As Fig. 7 and 8 show, on the 4th day after infecting, the GM-CSF values (586.7±95.5 pg/ml *vs*. 306.5±11.4 pg/ml, $p<0.05$) were significantly higher in the mice treated with ISL than in the control group.

On the 5th day after infecting, in the group administrated with ISL, the secretion level of a lot of the studied cytokines (at the time of the incubation for 24 hours and/or 48 hours) was significantly higher than in the control mice (Table 2):

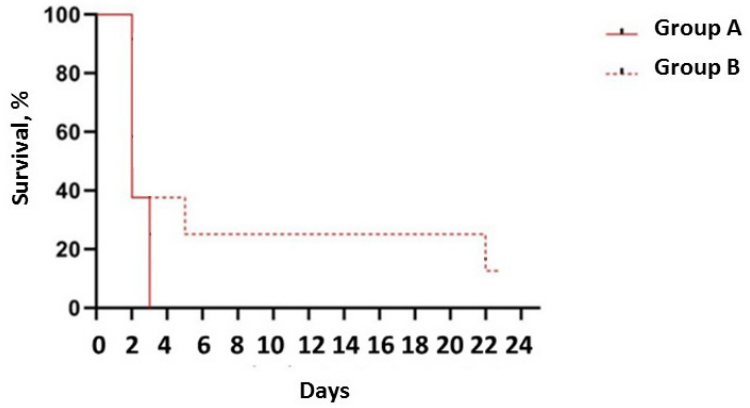

Figure 1 – Survival of Balb/C mice (males) infected with *S. aureus J49 ATCC 25923*

Note: Group A – control, 1.5–10^9 CFUs/mouse. Group B – a preliminary administration of ISL (30 mg/kg), 1.5×10^9 CFUs/mouse

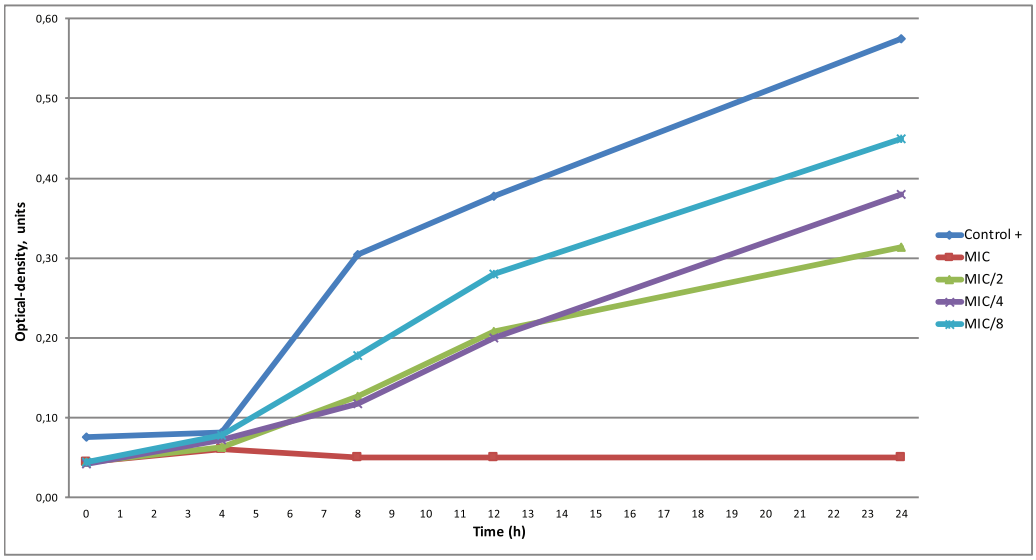

Figure 2 – Effect of ISL on the growth of *S. aureus J49 ATCC 25923*

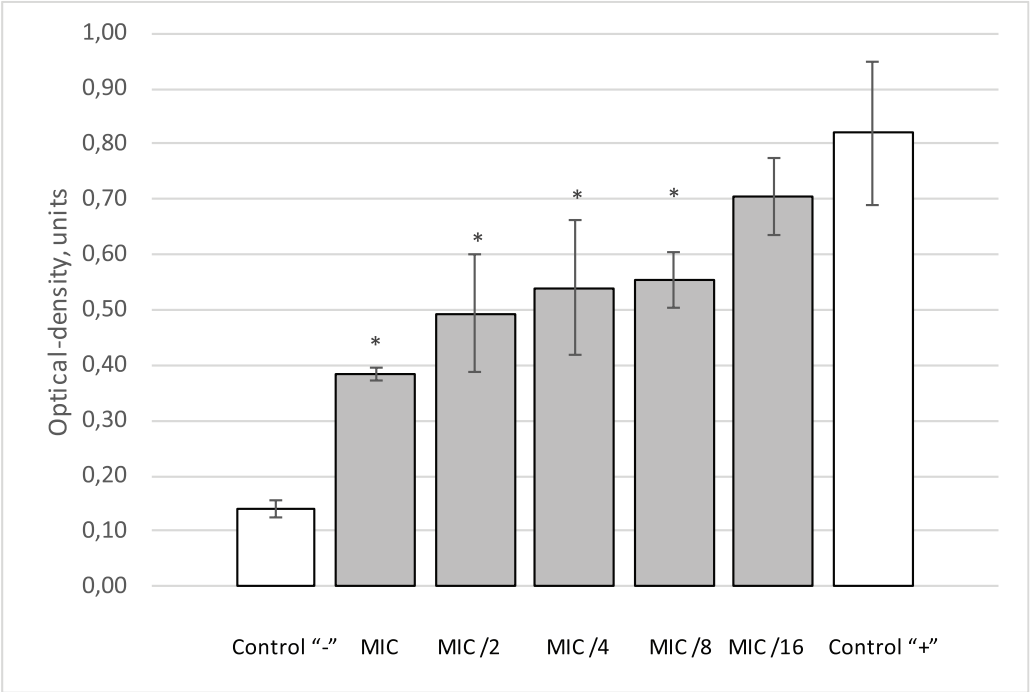

Figure 3 – Effect of ISL on biofilm formation of *S. aureus J49 ATCC 25923*

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DISCUSSION

The investigation is devoted to the study of the effects of the chalcone flavonoid ISL in the model of generalized staphylococcal infections. The aim of the paper was to study antibacterial and immunotropic mechanisms of ISL.

Despite the fact that mice's models of *S. aureus* infection weakly correlate with staphylococcal infection in humans [18], they are widely used in experimental medicine and pharmacology. It is known that very high inoculums are required to reproduce *S. aureus* infection in animals.

Thus, in the present work, generalized infections were reproduced in Balb/C mice by intraperitoneal injection of *S. aureus J49 ATCC 25923* suspension in the quantity of 5×10^8–1.5×10^9 CFUs/mouse. The generalization of the infection (sepsis) was confirmed by the presence of bacteremia, abscesses in internal organs even in the case of a bacterial load of 5×10^8 CFUs/mouse, which practically did not cause death of mice.

Mass mortality of the experimental animals was observed only at infecting 1.5×10^9 CFUs/mouse or more. Moreover, most of the mice died in the early stages of the infection (on days 2–3), which could indicate the development of a septic shock [19].

It was found out that the administration of ISL 1 hour before the bacterial infection significantly increased the survival rate of mice. In this case, the protective effect of ISL in staphylococcal sepsis could be realized by restraining the symptoms of a toxic shock, which are largely the result of the overproduction of cytokines (IL-2, INF-γ and TNF-α) [20] by T-lymphocytes activated by a superantigen [21].

In the culture of ConA-activated splenocytes, ISL dose-dependently inhibited the production of cytokines even at the concentrations which did not cause a decrease in proliferation. Thus, in the presence of ISL (4 μg/ml), the secretion of the entire spectrum of cytokines under study (IL-2, IL-12p70, IFNg, TNFα, IL-6, IL-22, IL-23, IL-17A, IL-17F, IL-17E/IL-25, GM-CSF, MIP-3a/CCL20, IL-10) was below the control values. When ISL was added to the lymphoid cell culture at the concentrations of 16-64 μg/ml, most cytokines (IL-2, IL-12p70, IFNg, TNFα, IL-23, IL-17A, IL-17F, IL-17E/IL-25, GM-CSF, MIP-3a/CCL20) were not detected.

Table 1 – Effect of ISL on cytokine secretion by splenocytes *in vitro*

	A, pg/ml	B, pg/ml
IL-2	<4.0	2020.0±115.7
IL-12p70	<7.9	23.8±2.6
IFNg	<4.5	6253.0±157
TNFa *	25.0±0.2	213.2± 16.1
IL-6 *	27.6±1.4	2249.0±132.0
IL-22*	13.2±0.4	1276.0±71.2
IL-23	<123.7	223.3±1.4
IL-17A	<20.4	724.5±76.9
IL-17F	<6.2.	968.4±107.4
IL-17E / IL-25	<377.5	527.6±25.4
GM-CSF	<22.5	310.8±32.6
MIP-3A / CCl20	<35.6	355.8±3.1
IL-10 *	32.8±0.1	424.3± 24.3

Note: A – the level of cytokines in the samples incubated with ISL (4 μg/ml); B – the level of cytokines in control samples. * – p <0.05

Table 2 – Effect of ISL on cytokines produced by splenocytes during staphylococcal infection in Balb/C mice, on the 5th day of incubation

	A, pg/ml	B, pg/ml
IL-2*	3818.0±265.9	2158.5±140.7
IL-12p70*	100.6±2.6	31.8±4.6
IFNg*	7191.0±0.0	2356.0±179.6
IL-22*	1028.5±33.2	604.4±45.4
IL-23*	374.0±17.8	186.3±0.0
IL-17A*	3094.5±95.5	756.9±20.3
L-17F*	1223.5±79.9	865.45±39.4
MIP-3a/CCL20*	428.8±16.7	302.0±0.8
IL-10*	664.0±23.7	56.0±2.7

Note: A – the group administrated with ISL (preliminary intraperitoneal injection, 10 mg/kg, three times); B – control group. * – p<0.05

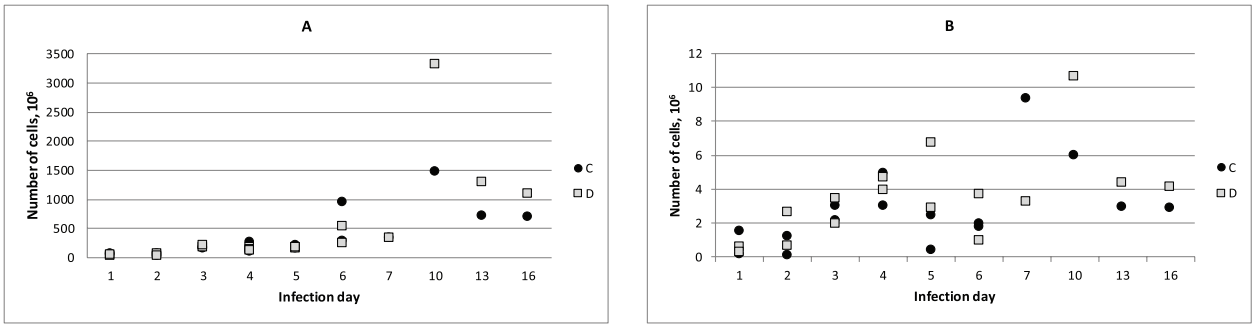

Figure 4 – Dynamics of splenocytes (A) and inguinal lymph node cells (B) in the model of Balb/C mice staphylococcal infection

Note: (C) Control group, 5×10^8 CFUs/mouse. (D) Pre-treatment with ISL (30 mg/kg), 5×10^8 CFUs/mouse. Intact mice: the number of splenocytes = $363.0\pm125.4\times10^6$ cells/mouse, the number of inguinal lymph node cells = $2.3\pm1.1\times10^6$ cells/mouse

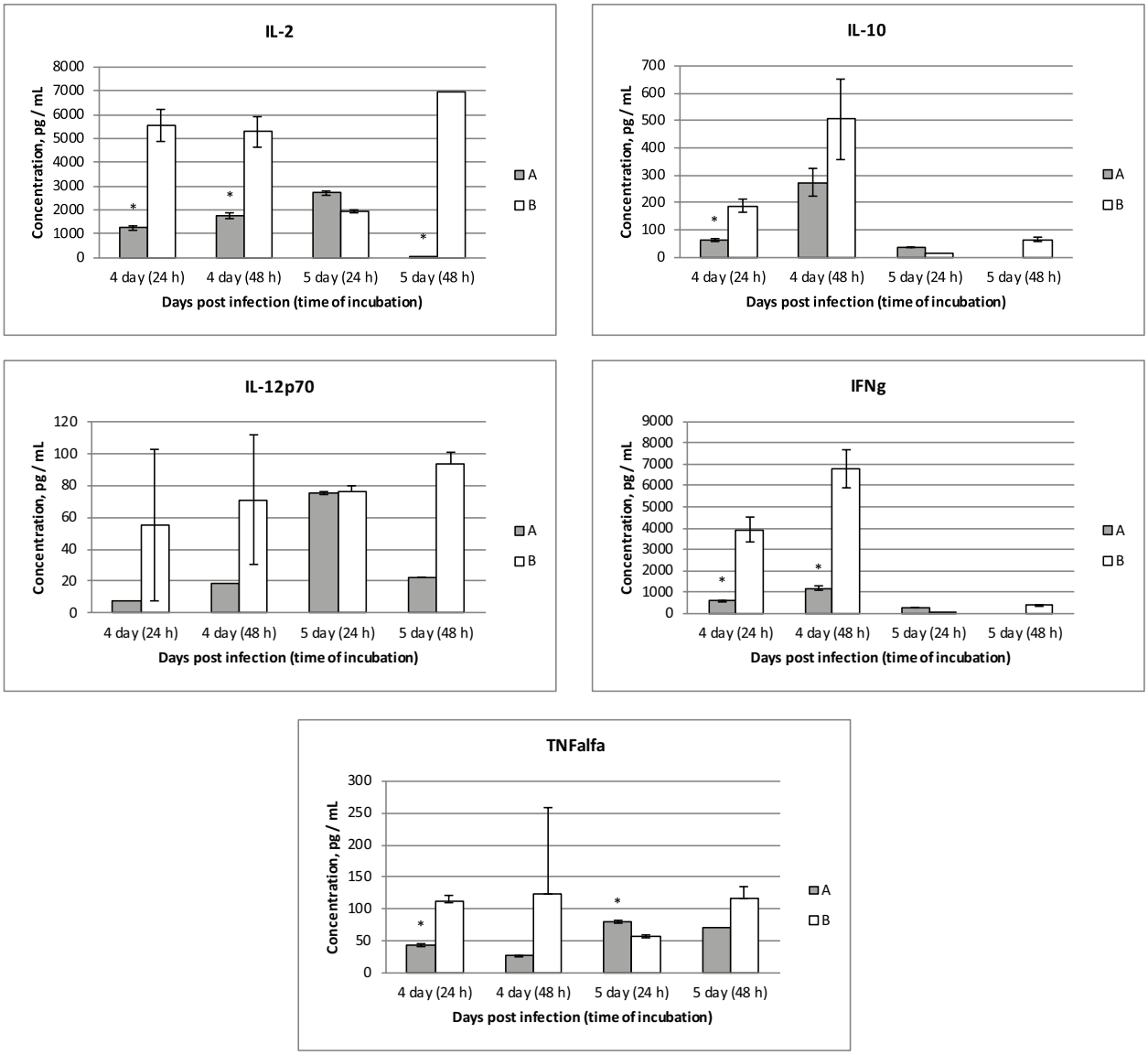

Figure 5 – Effect of preliminary ISL administration on the levels of cytokines (groups Th-1 and IL-10) produced by the cells of the inguinal lymph nodes of Balb/C mice infected with *S. aureus J49 ATCC 25923* (5×10^8 CFUs/mouse) (* $p<0.05$)

Note: (A) Preliminary ISL administration (30 mg/kg). (B) control group (* $p<0.05$)

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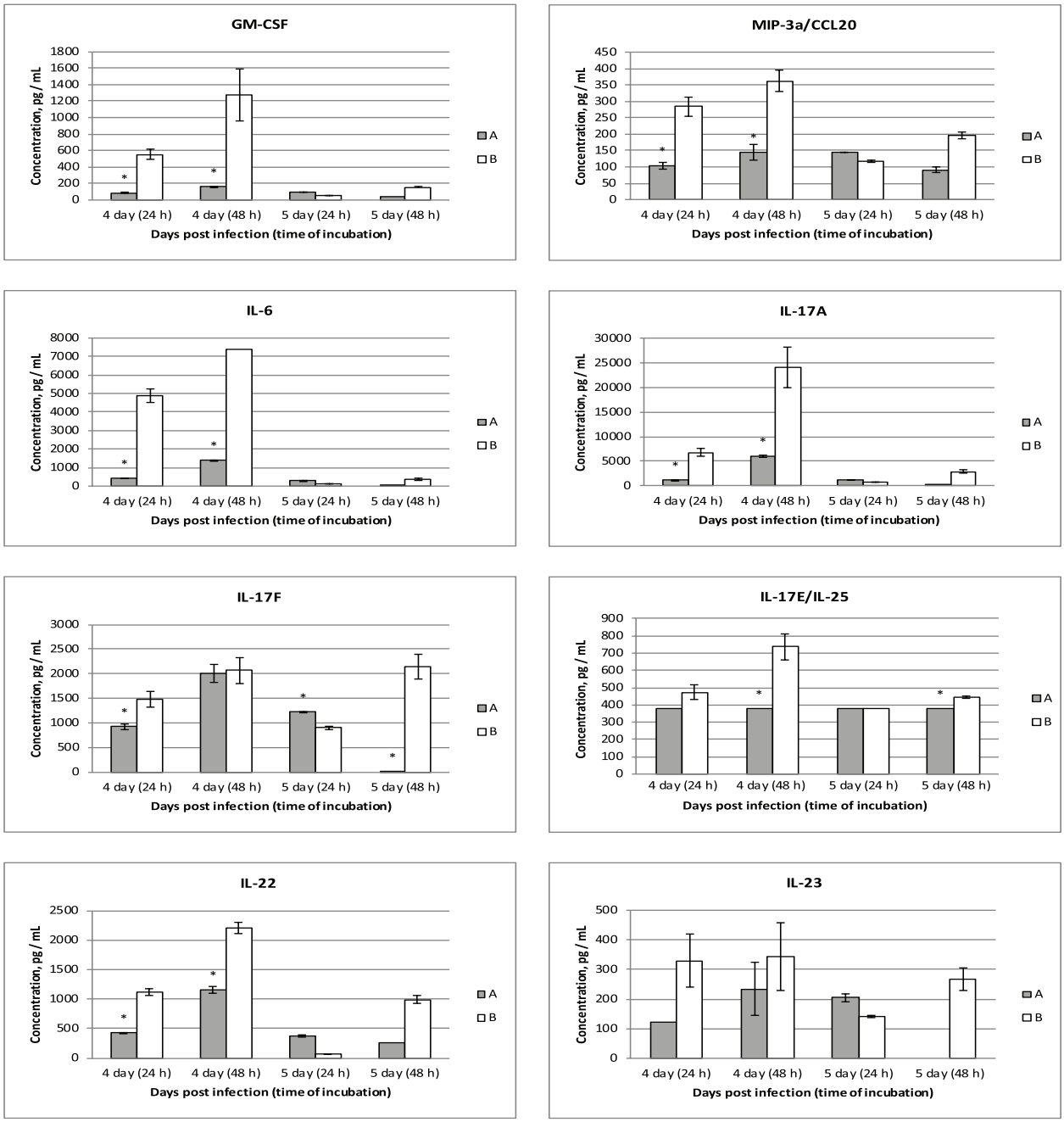

Figure 6 – Effect of preliminary ISL administration on the levels of cytokines (Th-17 group) produced by the cells of the inguinal lymph nodes of Balb/C mice infected with *S. aureus J49 ATCC 25923* (5×10^8 CFUs/mouse) (* p<0.05)

Note: (A) The group administrated with ISL (30 mg/kg). (B) Control group (* p<0.05)

Other researchers have also shown the effectiveness of ISL in the sepsis caused by other mechanisms. Thus, in the model of the sepsis caused by ligation and puncture of the cecum, ISL reduced the concentration of proinflammatory cytokines in the blood serum, the activity of NO-synthase, cyclooxygenase-2 [22], and also had antioxidant and anti-inflammatory effects [23].

In the early stages of the infection, the inflammatory response is mediated by the involvement of factors of innate, but not adaptive immunity. It is innate immune responses that are most significant for preventing generalization and limiting the purulent-inflammatory processes. According to the authors' opinions, it is positive that there is no suppressive effect of ISL on the migration of phagocytes to the focus of the pathogen introduction. In the present study, ISL did not reduce the number of cells after 24 h (chemotaxis of neutrophils) and 72 h (chemotaxis of macrophages) in response to the introduction of the inducer of migration, peptone. At

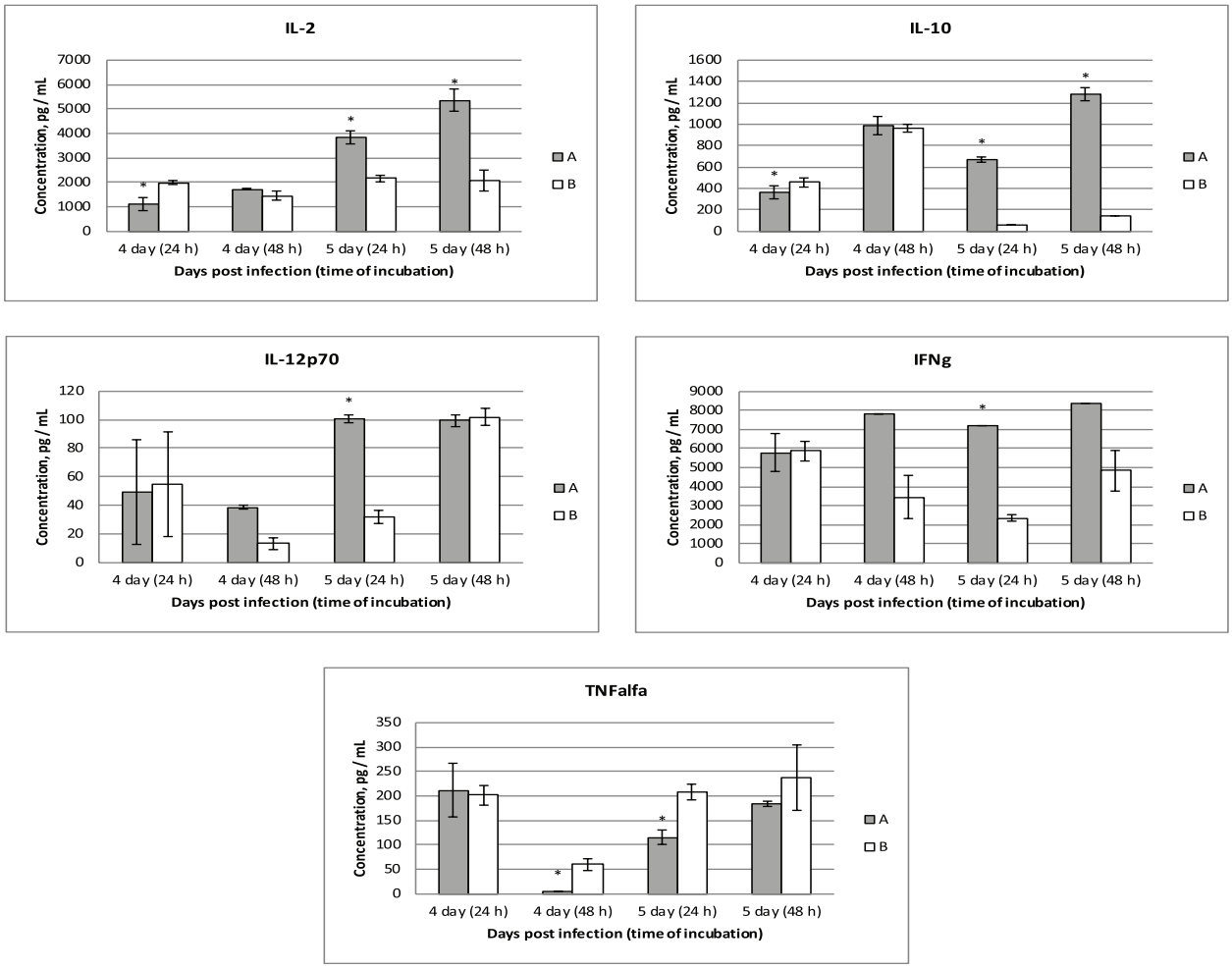

Figure 7 – Effect of preliminary ISL administration on cytokine levels (groups Th-1 and IL-10) produced by splenocytes of Balb/C mice infected with *S. aureus J49 ATCC 25923* (5×10^8 CFUs/mouse, intraperitoneal) (* p<0.05)

Note: (A) – the group administrated with ISL (30 mg/kg). (B) Control group (* p<0.05)

the same time, a visual assessment during autopsy of mice receiving ISL showed that, in comparison with the control group, a more pronounced adhesive process was observed with the formation of abscesses with dense capsules.

Based on the findings, as well as taking into account a rather short half-life of ISL [24], the authors can consider such substances being potentially of interest as an alternative to corticosteroids, preventing lethality in systemic inflammation.

In addition, ISL, in comparison with corticosteroids, has a direct antistaphylococcal effect. Under the conditions of the carried out experiment, the MIC of ISL against *S. aureus J49 ATCC 25923* was 64 μg/ml, which was comparable with the data of some authors who had studied the effect of ISL on other bacteria of the Staphylococcus genus [6]. Despite the fact that the antistaphylococcal activity was not high, ISL dose-dependently inhibited the suspension growth of *S. aureus J49 ATCC 25923* at the concentrations less than the MIC (8–32 μg/ml). It suggests that the antibacterial activity of ISL also played a role in increasing the survival of animals.

Staphylococcus bacteria are able to form biofilms on various surfaces. This ability of *S. aureus* was found out in both collection strains [25] and clinical isolates of *MSSA* and *MRSA* [26]. Using an atomic force microscopy and the MTT test, *S. aureus J49 ATCC 25923* strain was found out to be able to form biofilms on the plastic surface, and the addition of ISL at the concentrations lower than the MIC, inhibits the formation of bacterial biofilms. The antibiofilm effect of ISL was dose-dependent (4–32 μg/ml) and correlated with the severity of inhibition of the suspension growth of bacteria, which may indirectly indicate that this effect was a consequence of a direct antibacterial effect of ISL. The antibiofilm activity of ISL has been demonstrated by other researchers against *S. xylosus* [7]; however, the mechanisms of the ISL effect on the biofilms of the Staphylococcus genus bacteria have not been thoroughly studied, although there is information about other flavonoids that affect the *quorum sensing system* of *S. aureus.*

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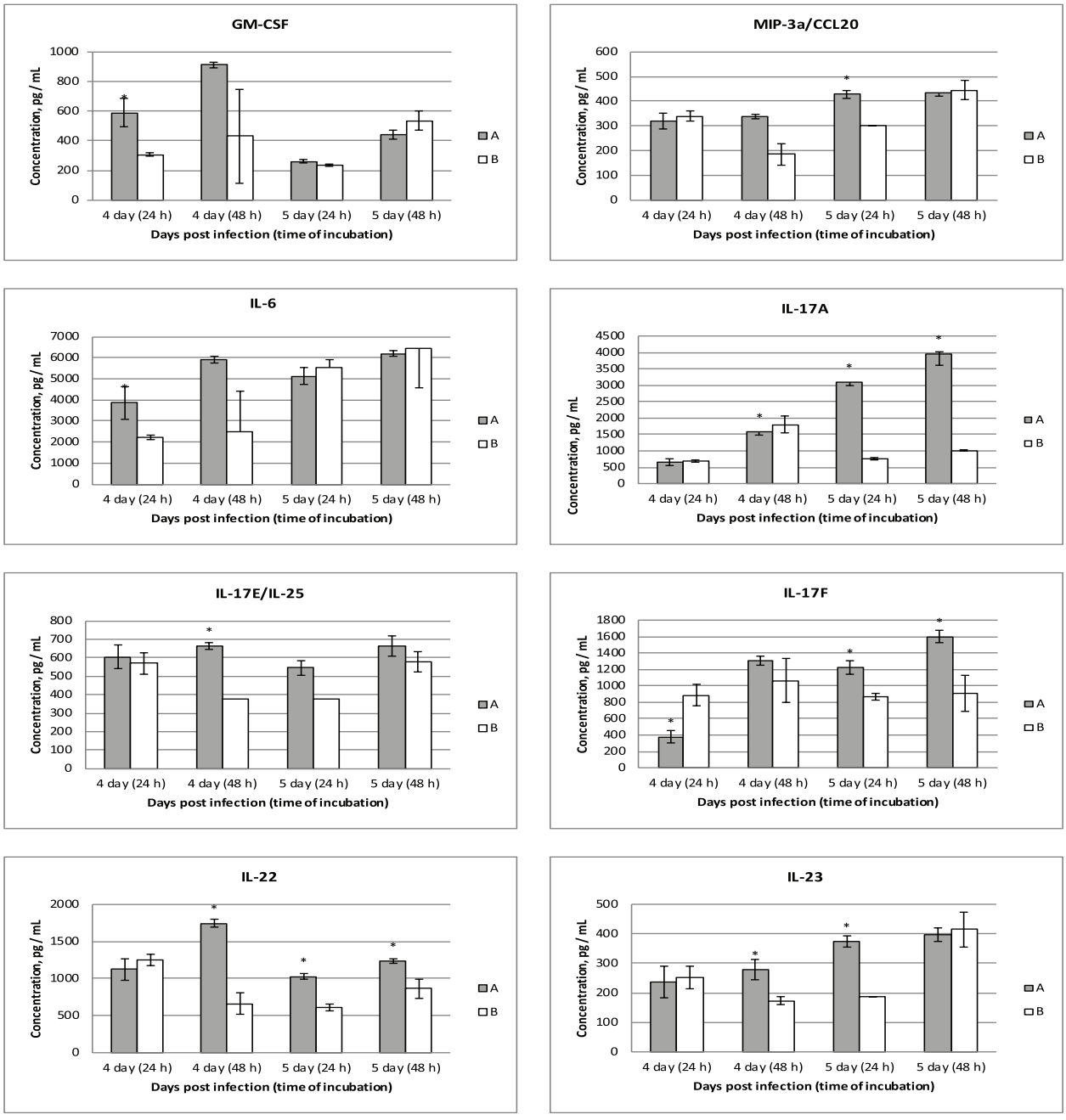

Figure 8 – Effect of preliminary ISL administration on cytokine levels (group Th-17), produced by splenocytes of Balb/C mice infected with *S. aureus J49 ATCC 25923* (5×10^8 CFUs/mouse, intraperitoneally) (*p<0.05)

Note: (A) – preliminary ISL administration (30 mg/kg). (B) Control group (* p<0.05)

In recent years, some publications have appeared about *S. aureus* actively avoiding immune surveillance, "turning off" various mechanisms of the adaptive immune response in the host organism [27]; it allows bacteria to persist even in the process of the development of an antigen-specific response.

To study the effect of ISL on the immune response, a mouse model of generalized infections was used, and no death of the animals was observed in the inoculation (5x10^8 CFUs/mouse). The dynamics of the number of cells of the inguinal lymph nodes and spleen, as well as their production of cytokines after infecting, was assessed.

Attention was drawn to the fact that in the first days after infecting the animals, the number of cells in the regional lymph nodes and spleen was significantly fewer than in the intact animals. Apparently, a massive bacterial invasion led to the development of a nonspecific response — a stress response, as a result of which the increased secretion of corticosteroids in the first days of the septic process, could be the cause of the lymphocytic effect [28]. Only after 3 days of infecting, there

was a gradual increase in the number of lymphocytes in the regional lymph nodes, reaching maximum values on day 7 (in the control group) and on day 10 (in the group administrated with ISL). To study the secretion of cytokines, regional lymph nodes and spleen from mice were removed on the 4th and 5th days after infecting (the period of increasing "cellularity").

When lymphocytes are activated, first IL-2 is produced, and then other cytokines necessary for the differentiation of various Th-subpopulations. The functional state of Th-subpopulations is usually judged by the production of a characteristic spectrum of cytokines by immunocompetent cells: IFNg is a Th1 marker, IL-4 is a Th2 marker, and IL-17A is the main Th17 cytokine.

From the point of view of modern concepts, Th17 cells are involved in antistaphylococcal immunity, enhancing the effector function of neutrophils [29] and, thus, acting as the most important protective population. However, taking into consideration the plasticity of Th-subpopulations in a dynamically changing microenvironment *in vivo*, cytokines of Th17-dependent effectors such as GM-CSF, MIP-3a/CCL20, are also evaluated.

The study of supernatants of the cells of the inguinal lymph nodes of the infected control mice, revealed the predominance of such cytokines as IL-12p70, IFNg, IL-6, IL-22, IL-23, IL-17A. IL-17F, IL-17E/IL-25. This could indicate the differentiation of activated $CD4^+$-cells into Th1 and Th17. The differentiation hypothesis is also supported by an increase in serum concentrations of GM-CSF, as well as the antibacterial chemokine MIP-3a/CCL20. The soluble components of *S. aureus* contribute to the induction of Foxp3+Treg, and in addition to an increase in the secretion of proinflammatory cytokines in the control group, an increase in the secretion of IL-10 was noted. It was probably produced by T-regulatory cells that suppress excessive inflammation by suppressing Th1 and Th17. In the group of mice treated with ISL, the suppression of the secretion of both pro-inflammatory (IL-2, IFNg, IL-6, IL-17A, GM-CSF) cytokines and IL-10 by cells of the inguinal lymph nodes was revealed, the concentration of which was less than the control values by more than twice.

In the present experiment, ISL, like many other flavonoids [30], exhibited immunosuppressive properties. In the culture of mononuclear cells activated by the T-cell mitogen KonA, ISL dose-dependently inhibited proliferation: in the concentration of 8 μg/ml, the proliferation was suppressed by about twice, while in the concentrations above 16 μg/ml, the proliferative response was almost completely absent, and it reduced the cytokine production not only *in vitro,* but also by inguinal lymph node cells in the infected mice. Thus, despite the presence of antibacterial mechanisms, ISL could potentially provoke the generalization of the infection. However, when blood was inoculated on the first day of infecting, bacteremia in the group of the control mice was significantly higher and reached 10^5 CFUs/ml, while against the background of the introduction of ISL on a dense medium, single colonies grew, indicating bacteremia $<10^3$ CFUs/ml, on the following days, for a week, as shown by the blood culture, bacteremia was not detected in all animals in both groups at low levels ($<10^3$ CFUs/ml).

Considering that the spleen plays an important role in curbing the hematogenous spread of infection [31], the production of cytokines by splenocytes was investigated 4-5 days after infecting, where unexpected results were obtained. In the spleens of the both groups, the maximum "cellularity" was observed on the 10th day of infecting, but in the group administrated with ISL, their number was twice higher than the control values. Splenocytes of mice, which were injected with ISL before infecting, significantly increased the production of cytokines that activate the immune response in the Th-1 type (IL12p70, TNFα, IFNg) and Th-17 (IL22, IL23, IL6, IL17). It has been suggested that the expansion of a large number of T-cells after the stimulation with a superantigen, can deplete IL-2, thereby limiting the development of a protective T-cell response [32]. It is possible that the inhibition of the cytokines secretion (in particular, IL-2) and the lower bacterial load against the background of the ISL administration promoted a more effective participation of splenocytes in the immune response.

Against the background of the ISL administration, attention is drawn to a more effective Th-17 response with an increase in cytokines of innate immunity effectors (GM-CSF, MIP-3a/CCL20). It has been found that chemokines such as MIP-3a/CCL20, have a pronounced antibacterial activity against both gram-positive and gram-negative bacteria [33]. It is assumed that pro-inflammatory Th17 cells of the first wave actively secrete IL-17 in the target tissues; it induces the secretion of the antibacterial peptide MIP-3a/CCL20 by a variety of cells. It is likely that the insufficient activity of MIP-3a/CCL20 can lead to a decrease in the T-cell-mediated control of bacterial pathogen eradication.

CONCLUSION

Thus, in the course of the study, it was revealed that the preliminary administration of ISL increases the survival rate of the mice in the generalized infection caused by *S. aureus J49 ATCC 25923.* This protective effect of ISL is based on both antimicrobial (moderate direct antistaphylococcal with MIC = 64 μg/ml, antibiofilm in the concentrations below MIC), and immunomodulatory defense mechanisms. All these factors deserve attention in order to create new drugs that reduce mortality in sepsis.

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AUTHORS' CONTRIBUTION

All authors equally contributed to the research work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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