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EXPERIMENTAL BIOLOGY

Revisiting the Problem of Finding Effective Sepsis Treatment Solutions

M. N. Cherkasova[1], V. N. Laskavy[2], T. G. Borovaya[1], V. G. Zhukhovitsky[1,3], and O. M. Sednev[2]

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We studied the effect of a preparation containing ultralow doses of formic aldehyde on the course of experimental sepsis caused by intraperitoneal injection of two different strains of *Pseudomonas aeruginosa* (1623 and 5266) to C57BL/6 male mice. Microscopy and quantitative bacteriological tests in the dynamics of the infectious process demonstrated a positive effect of the drug: 100% survival of animals, preserved histological structure of the studied organs (lungs, liver, kidneys, spleen, and adrenal glands), a sharp decrease in the level of contamination of the blood and organ homogenates during the first hours after infection, and complete absence of bacteria in inoculates on day 7 after infection. These findings suggest the effectiveness of ultralow doses of formic acid aldehyde in the composition of the medicinal product in the treatment of experimental sepsis caused by *P. aeruginosa* strains 1623 and 5266 in mice.

Key Words: *sepsis; Pseudomonas aeruginosa; formic acid; pulmonary acinus; renal tubule*

In the search for effective means of combating sepsis, an important role is given to experimental modeling, which makes it possible to study sepsis in dynamics, from the earliest stages (which is practically impossible in a clinical setting), and to assess the effectiveness of various drugs.

During the Great Patriotic War (1941-1945), Kalceks preparation was recommended by the Sanitary Directorate of the Red Army for the prevention of pneumonia and septic complications among personnel. In the post-war period, this cheap domestic drug with hexamethylenetetramine as an active principle was used for a long time for the prevention and treatment of viral and bacterial infections, but was later displaced by new generation anti-inflammatory and immunomodulatory agents. The therapeutic and preventive effect of hexamethylenetetramine is based on the fact that in the acidic environment that occurs in tissues during inflammation it decomposes with the formation of formic acid aldehyde that causes destruction of pathogens. The current high relevance of the problem of sepsis as an uncontrolled multi-organ inflammatory process requires an active search for effective means to combat it. We considered it expedient to test the effect of a preparation containing microdoses (0.073-0.075%) of formic acid aldehyde in an isotonic solution within the framework of the mouse sepsis model developed by us earlier.

The aim of the study was to analyze the structural and bacteriological characteristics of internal organs of septic mice treated with a preparation containing ultralow doses of formic acid aldehyde.

[1]N. F. Gamaleya National Research Center of Epidemiology and Microbiology, Ministry of Health of the Russian Federation, Moscow, Russia; [2]LLC "Research Institute of Medical and Veterinary Biotechnologies", Moscow, Russia; [3]Russian Medical Academy of Continuous Professional Education, Ministry of Health of the Russian Federation, Moscow, Russia. ***Address for correspondence:*** mari.cherkasova.1991@bk.ru. M. N. Cherkasova

MATERIALS AND METHODS

Sepsis was modeled on male C57BL/6 mice. The experiments were carried out in accordance with the Directive 2010/63/EU of the European Parliament and of the Council (September 22, 2010; On the Protection of Animals Used for Scientific Purposes). Animals of group 1 (model 1623, n=60) were injected once intraperitoneally with a daily broth culture of the *P. aeruginosa* 1623 strain isolated from the bronchial lavage of a patient who was on mechanical ventilation; the infectious dose was 7×10^6 CFU/mouse in a volume of 0.5 ml. Animals of group 2 (model 5266, n=60) were injected once intraperitoneally with a daily broth culture of *P. aeruginosa* 5266 strain isolated from the wound discharge of a patient with burn disease; the infectious dose was 3×10^7 CFU/mouse in a volume of 0.5 ml. The choice of *P. aeruginosa* as the causative agent of the infectious process was dictated by the dominance of this bacterium in the etiology of hospital sepsis, primarily in patients in intensive care and burn departments; *P. aeruginosa* strains 1623 and 5266 differed in the profiles of produced exotoxins: $ExoA^+$, $ExoY^+$, $ExoT^+$, $ExoS^+$, $ExoU^-$ and $ExoA^+$, $ExoY^+$, $ExoT^+$, $ExoS^-$, and $ExoU^+$, respectively. The blood, liver, kidneys, spleen, lungs, and adrenal glands were examined. After infection, each group of animals was divided into two subgroups. Mice of the 1st subgroup (24 animals in each model) did not receive treatment. Mice of the 2nd subgroup (36 animals in each model) received an intramuscular injection (into the right paw) of a product with laboratory name Astrabionorm [1] in a volume of 0.2 ml 1 h after infection and again 1.5 h later. Four hours after infection, biomaterial was taken for histological analysis from 6 animals of the 1st and 2nd subgroups in each model, and material for bacteriological examination was taken from another 6 animals of these subgroups. The remaining mice of the 2nd subgroup (24 h in each model) were given repeated doses of Astrabionorm 7 and 24 h after infection and then, the biomaterial for histological analysis (6 mice in each model) and for bacteriological examination (6 mice in each model) were taken 24 h after infection. The remaining animals of the 2nd subgroup (n=12 in each model) were further monitored. These mice were in a clinically healthy state, and the corresponding biomaterial was obtained 1 week after infection. The remaining untreated mice (n=12 in each model) reached the terminal stage of the septic process by 24-26 h after infection: for histological analysis, biomaterial was taken from 6 animals (in each model), for bacteriological analysis, from another 6 animals (in each model).

Histological processing of the biomaterial was carried out according to generally accepted methods. Serial sections of organs (4-5 μm thick) were stained with hematoxylin and eosin, analyzed, and photographed using an AxioPlus light microscope (Carl Zeiss). To determine the dynamics of the bacterial load, weighed portions of organ samples were homogenized in sterile porcelain mortars, a series of 10-fold dilutions was performed and inoculated into plates with Columbia Agar Base nutrient medium (Becton Dickinson). The plates with inoculations were cultured for 24 h at 37°C, after which the number of grown colonies of *P. aeruginosa* was counted with determination of the mean values (CFU/g) 4, 24 h, and 7 days after infection. Simultaneously, peripheral blood (0.5 ml) was cultured and *P. aeruginosa* colonies (CFU/ml) were counted in the dynamics of sepsis.

Before the main part of the experiment, Astrabionorm was administered intramuscularly to a small group of mice (n=6) three times within 24 h in a volume of 0.2 ml, to exclude the toxic effect of the drug. No damage to skeletal muscle tissue was found in the area of drug administration, all animals remained healthy during the entire observation period (7 days), histological examination of organs obtained from two animals of this group revealed no pathological changes.

Statistical processing of the obtained results was carried out in Microsoft Excel 2010. The significance of differences between the groups was assessed by Student's t test. The data are presented as $M\pm SEM$. The differences were considered statistically significant at $p<0.05$.

RESULTS

In all animals of the 1st subgroup in both models, signs of illness appeared 4 h after infection (the mice crawled slowly, were disheveled, *etc.*). Histological changes in the studied visceral organs in untreated animals in both models were similar: they appeared within the first hours after infection and became more pronounced by the terminal stage that occurred in 24-26 h after infection in all mice of models 1623 and 5266. In histological sections of the lungs of untreated mice, "hyper-aerated" areas (Fig. 1, *a*) with "broken" acini were seen along with non-aerated zones of interstitial inflammation 4 h after infection. This combination of histological patterns is apparently a result of a difficult lung excursion and severe respiratory failure (which was also observed visually). The degree of contamination of the lungs with the pathogen was high, especially in the 1623 model (Table 1). The described signs of pulmonary changes were aggravated by the terminal stage of sepsis, when the number of pathogen colonies in model 1623 increased by almost 1.5 times, while in model 5266,

it was one order of magnitude higher than in model 1623 (Table 1).

Contamination of the lungs in animals of the 2nd subgroup in both models treated with Astrabionorm progressively decreased, and by day 7 of observation, only single colonies were detected. The histostructure of the lungs corresponded to normal (Fig. 1, *b*) during the entire observation period. In the 1st subgroup, a consistently high growth of colonies of *P. aeruginosa* 1623 and 5266 from the liver and kidney homogenates was recorded in both models (Table 1) with the maximum value in model 5266 during the terminal stage. In the liver of mice of the 1st subgroup (Fig. 2, *a*), in contrast to the 2nd subgroup (Fig. 2, *b*), we observed small foci of leukocyte infiltration, signs of hepatocyte destruction, venous stasis and thrombosis that increased by the terminal stage; in the kidneys, destructive changes in the proximal tubules prevailed (Fig. 3, *a*), deformation of the nephron bodies, and pyknosis of the capillary glomerular cells (Fig. 3, *b*). In treated mice of the 2nd subgroup (in both models), a significantly lower growth of *P. aeruginosa* from liver and kidney homogenates was recorded, especially in model 5266 by 24 h of observation (Table 1). Histological changes in the spleen in animals of the 1st subgroup with models 1623 and 5266 were somewhat different: in the first model, almost all mice had signs of white pulp hyperplasia at the studied periods of sepsis development; in model 5266, where the degree of seeding of the spleen reached higher values (Table 1), most mice showed signs of white pulp hypoplasia.

In the 2nd subgroup, administration of the drug reduced activity of *P. aeruginosa* colony formation from spleen homogenates, which was especially significant in both models by 24 h of observation. The histological picture of the spleen in mice of the 2nd subgroup in both models corresponded to the physiological norm [2]. The growth of *P. aeruginosa* colonies from adrenal homogenates compared with other organs in mice of the 1st subgroup in both models was not high (Table 1), and in mice of the 2nd subgroup in both models, a very significant decrease in inoculation was observed by 24 h of observation. Structural changes in the adrenal glands during sepsis in a mice of the 1st subgroup in both models were similar and consisted in reduction of the zona glomerulosa and zona fasciculata of the cortex and medulla, degeneration of corticosterocytes of the zona glomerulosa, the columnar part of the zona fasciculata, and chromaffinocytes of the medulla (especially pronounced by 24 h of observation), expansion of the paramedullary part of the zona fasciculata, which does not have columnar architectonics and does not show histological signs of steroid activity. In the 2nd subgroup,

TABLE 1. Effect of Astrabionorm on the Course of Experimental Sepsis in Mice (*M*±*SD*)

Model		Blood, CFU/ml	Liver, CFU/g	Kidneys, CFU/g	Spleen, CFU/g	Lung, CFU/g	Adrenal glands, CFU/g
Model 1623, 4 h	subgroup 1	$7.5\times10^5\pm3.3\times10^4$	$3.8\times10^5\pm1.4\times10^4$	$5.0\times10^5\pm5.4\times10^4$	$2.7\times10^5\pm3.6\times10^4$	$4.2\times10^5\pm2.4\times10^3$	$5.3\times10^4\pm5.3\times10^3$
	subgroup 2	$8.0\times10^2\pm1.9\times10^{2***}$	$1.2\times10^3\pm8.8\times10^{1***}$	$1.0\times10^5\pm1.3\times10^{4***}$	$4.7\times10^5\pm2.9\times10^{4***}$	$2.6\times10^4\pm1.6\times10^{3***}$	$1.9\times10^4\pm2.2\times10^{3**}$
Model 1623, 24 h	subgroup 1	$2.5\times10^5\pm8.2\times10^3$	$1.5\times10^5\pm2.9\times10^3$	$5.4\times10^5\pm3.1\times10^4$	$5.0\times10^5\pm3.2\times10^4$	$6.1\times10^5\pm5.6\times10^4$	$1.8\times10^5\pm1.3\times10^4$
	subgroup 2	$5.0\times10^3\pm3.9\times10^{2***}$	$1.2\times10^3\pm1.8\times10^{2***}$	$1.9\times10^3\pm2.2\times10^{2***}$	$8.3\times10^2\pm4.1\times10^{1***}$	$8.3\times10^2\pm2.9\times10^{1***}$	$3.7\times10^2\pm4.3\times10^{1***}$
Model 5266, 4 h	subgroup 1	$4.0\times10^4\pm2.3\times10^3$	$1.4\times10^5\pm9.5\times10^3$	$5.1\times10^5\pm3.5\times10^4$	$8.5\times10^6\pm4.9\times10^4$	$2.4\times10^5\pm3.8\times10^3$	$6.9\times10^4\pm8.8\times10^3$
	subgroup 2	$2.2\times10^4\pm1.1\times10^{3**}$	$2.2\times10^4\pm1.1\times10^{3***}$	$9.7\times10^4\pm7.5\times10^{3***}$	$9.2\times10^4\pm7.5\times10^{3***}$	$3.3\times10^4\pm3.0\times10^{3**}$	$5.7\times10^4\pm4.0\times10^3$
Model 5266, 24 h	subgroup 1	$1.5\times10^6\pm1.7\times10^5$	$1.4\times10^6\pm4.4\times10^4$	$5.4\times10^6\pm5.6\times10^5$	$7.1\times10^6\pm6.4\times10^5$	$5.9\times10^6\pm5.7\times10^5$	$3.5\times10^5\pm2.2\times10^4$
	subgroup 2	$8.0\times10^2\pm8.0\times10^1$	$3.6\times10^2\pm4.1\times10^{1***}$	$2.2\times10^3\pm1.5\times10^{2***}$	$5.8\times10^3\pm7.1\times10^{2***}$	$1.4\times10^3\pm2.7\times10^{2***}$	$4.6\times10^2\pm5.7\times10^{1***}$
Model 1623, 7 days[1]	subgroup 2	0	1	3	0	10	0
Model 5266, 7 days[1]	subgroup 2	1	0	0	0	5	0

Note. [1]CFU/dish. $^{**}p<0.01$, $^{***}p<0.001$ in comparison with the 1st subgroup.

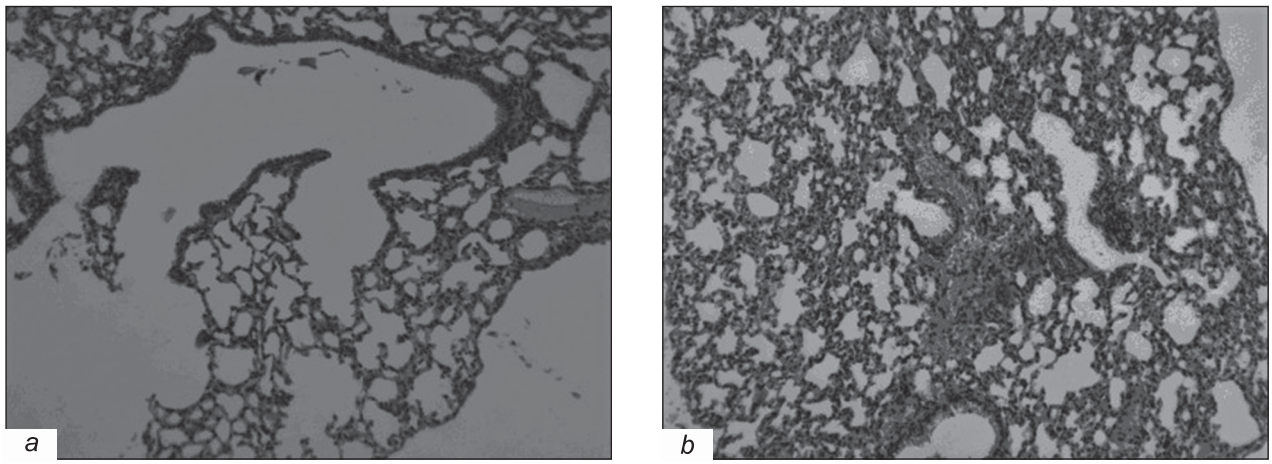

Fig. 1. A fragment of the lung of a mouse of the 1st subgroup 4 h after infection (*a*) and a mouse of the 2nd subgroup 24 h after infection (*b*) *P. aeruginosa* 1623. Hematoxylin and eosin staining, ×100. *a*) Ruptures of pulmonary acini and bronchioles; *b*) histostructure of the lung corresponds to normal.

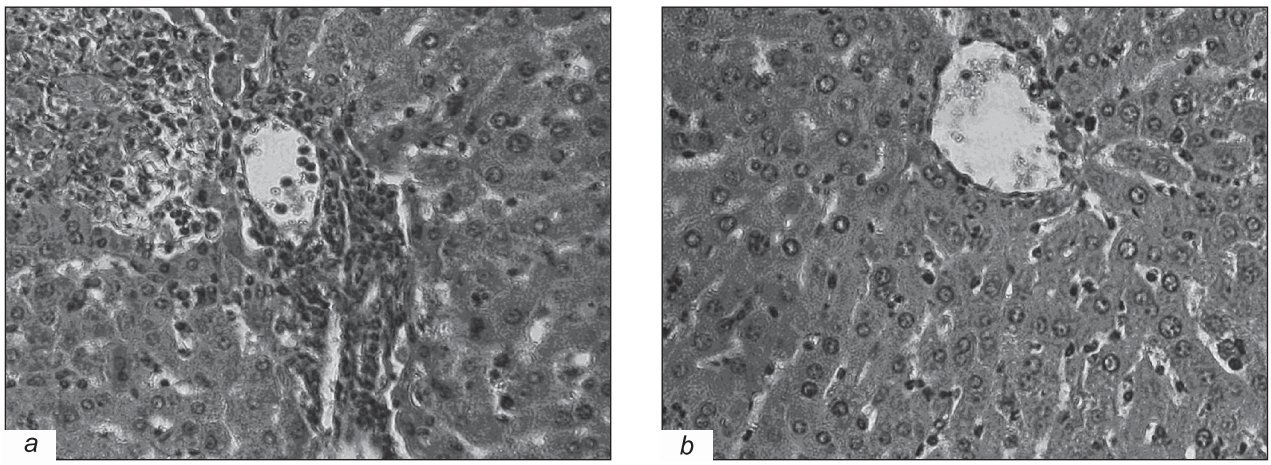

Fig. 2. A fragment of the liver parenchyma of a mouse of the 1st subgroup (*a*) and the 2nd subgroup (*b*) 4 h after infection with *P. aeruginosa* 5266. Hematoxylin and eosin staining, ×200. *a*) A site of destruction in the parenchyma, accumulation of lymphocytes in the region of the portal tract; *b*) preserved cytoarchitectonics of the hepatic lobule.

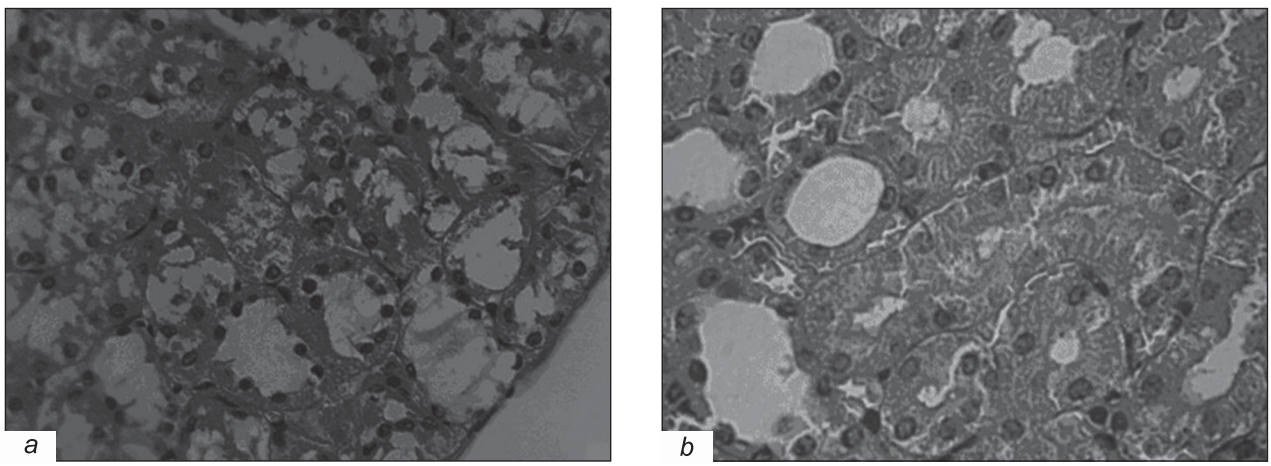

Fig. 3. A fragment of the cortical substance of the mouse kidney of the 1st subgroup (*a*) and the 2nd subgroup (*b*) 4 h after infection *P. aeruginosa* 1623. Hematoxylin and eosin staining, ×200 (*a*), ×400 (*b*). *a*) Destructive changes in the nephron tubules; *b*) the structure of nephron tubules corresponds to the norm.

pronounced hypertrophy of corticosterocytes of the zona fasciculata was observed in both models.

Seven days after infection, the results of culturing of blood samples and organ homogenates of all remaining animals of the 2nd subgroup were negative in both models (Table 1).

Widely used experimental models of sepsis, introduction of LPS (a component of the cell wall of gram-negative bacteria), caecal ligation with subsequent microperforation of its wall, and others, do not fully reflect the natural course of sepsis in humans. LPS is not the only, though undoubtedly powerful, antigen of gram-negative bacteria, and the caecal ligation model represents only an experimental variant of sepsis in peritonitis. The model of sepsis that we created by intraperitoneal injection of a live pathogen *P. aeruginosa* is also not a morphogenetic equivalent of this pathology in humans, however, like the above models, to a certain extent it corresponds to the three pathological criteria [3] adopted for sepsis in humans. Due to the absence of microscopic criteria for changes in internal organs that are natural for sepsis of various etiologies, when assessing the adequacy of the created model, one can only conditionally focus on three main pathological signs [3]: the presence of a primary septic focus of infection in the body (in our model, such a focus was created by introducing into the abdominal cavity a certain dose of the pathogen), the presence of secondary septic foci (this corresponds to inoculation of large amounts of the pathogen from organ homogenates and morphological changes in these organs), the reaction of the white pulp of the spleen (in our model, signs of hyperplasia of the white pulp of the spleen and depletion of the white pulp followed by hypoplasia in animals with the highest contamination of the organ with *P. aeruginosa*). Finally, the last argument in favor of the adequacy of the model can be considered the early appearance of bacteremia and dense growth of *P. aeruginosa* colonies from organ homogenates within the first hours after infection, as well as 100% death of untreated animals.

As for the drug used in the experiment, it should be noted that formic acid aldehyde (the principal component of Astrabionorm) is synthesized in living organisms, *i.e.* it is a natural metabolite involved in many biological processes [4]. In the body, its physiological level is constantly maintained due to the action of oxidative enzymes: P450 monooxygenases, mitochondrial ALDH2, and formaldehyde dehydrogenase [5-7]. Formic acid aldehyde is found in all internal fluids of humans and animals, and its certain concentration is important for vital activity of cells, although in higher concentrations it can also act as a genotoxin [8].

Many viruses are sensitive to ultralow doses of formic acid aldehyde and the concentration used does not have a toxic effect on cells [1].The results obtained by us (the preservation of the physiological histostructure of visceral organs in infected animals, the elimination of the pathogen from the blood and organs that occurs within the first hours of the development of the septic process, and the almost complete absence of pathogen in inoculates of the blood and organ homogenates on day 7 after infection) suggest the effectiveness of ultralow doses of formic acid aldehyde in the composition of "Astrabionorm" in the treatment of experimental sepsis caused by strains of *P. aeruginosa* 1623 and 5266 in mice.

Although the results obtained in the course of studying the experimental models cannot be unambiguously extrapolated to human sepsis, they allow us to raise the question of the feasibility of conducting preclinical trials of the drug Astrabionorm using microorganisms of various groups as inducers of the septic process: enterobacteria, gram-positive bacteria, and fungi.

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