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Саногенетический эффект лимонной и янтарной кислот в условиях воздействия инактивированных *M. tuberculosis* на крыс

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Резюме

Введение. Поиск способов коррекции патогенетических нарушений, обусловленных воздействием возбудителя социально значимого заболевания – *M. tuberculosis* на организм, определяет актуальность проведенного исследования и его цель: изучение роли лимонной и янтарной кислот в защитно-приспособительных процессах на фоне индуцированной инактивированными микобактериями патологии соединительной ткани у теплокровных животных.

Методы. Исследования проведены на крысах линии *Wistar*. На фоне индуцированной адъювантом Фрейнда (АФ, водно-масляная эмульсия термически обработанных микобактерий туберкулеза) патологии крысам вводили с едой смесь органических кислот в минимальной (17 мг/кг м.т.) и максимальной (88 мг/кг м.т.) дозировке на протяжении 4 недель. Гематологические и биохимические исследования проводили стандартными методами. Активность сукцинатдегидрогеназы (СДГ) в лимфоцитах крови определяли цитобиохимическим методом. Рентгеновские снимки получали на стационарном ветеринарном аппарате.

Результаты. На фоне сформированной АФ патологии (лейкоцитоз (увеличение лейкоцитов на 28 % относительно негативного контроля, $p < 0,05$), окислительный стресс (рост содержания малонового диальдегида (МДА) на 40 %, $p < 0,001$; ингибирование каталазы на 4 %), субхондральный склероз головок костей), животные в условиях защитного воздействия карбоновыми кислотами характеризуются дозозависимым купированием иммунотоксических признаков заболевания (нормализация численности лейкоцитов ($p < 0,05$ относительно модельных животных); снижение МДА на 27 %, $p < 0,001$, активация каталазы на 10 %, $p < 0,01$; нормализация СДГ; снижение дистрофических изменений в суставном аппарате животных).

Заключение. Результаты гематологических, биохимических и рентгенологических исследований свидетельствуют о возможности модификации цитрат-сукцинатной смесью патобиохимических и патоморфологических изменений, обусловленных введением инактивированных *M. tuberculosis* теплокровным животным, что позволяет глубже раскрыть патогенез и повысить эффективность проводимой терапии.

Ключевые слова: активность митохондрий, воспалительный процесс, лимонная кислота; микобактерии, патология соединительной ткани, перекисное окисление липидов, резистентность к инфекту, саногенез туберкулеза, тканевое дыхание, янтарная кислота.

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Therapeutic Effects of Citric and Succinic Acids in Rats Exposed to Inactivated *M. tuberculosis*

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Summary
Introduction. The search for methods of correcting pathogenetic disorders related to *Mycobacterium tuberculosis*, the causative agent of tuberculosis, a highly hazardous communicable and socially significant disease, determines the relevance of the research and its *objective* to study the role of citric and succinic acids in protective and adaptive processes in warm-blooded animals with connective tissue disorders induced by inactivated mycobacteria.
Materials and methods. The study was conducted on male *Wistar* rats with diseases induced by complete Freund's adjuvant (a mineral oil emulsion containing heat-killed *Mycobacterium tuberculosis*). The animals were given a feed-added mixture of organic acids at 17 mg/kg body weight (minimum) and 88 mg/kg body weight (maximum) for 4 weeks. Hematology and biochemistry tests were performed using standard methods. The activity of succinate dehydrogenase in blood lymphocytes was determined by the cytobiochemical method. X-rays were obtained using stationary veterinary imaging equipment.
Results. The protective effect of carboxylic acids in the exposed animals with Freund's adjuvant-induced leukocytosis (expressed by a 28 % increase in white blood cells compared to the negative control, $p < 0.05$), oxidative stress (expressed by an increase in the concentration of malondialdehyde (MDA) by 40 %, $p < 0.001$, and in inhibition of catalase by 4 %), and subchondral bone sclerosis was characterized by a dose-dependent reduction in immunotoxic manifestations of the disease such as normalization of the number of white blood cells ($p < 0.05$ compared to model animals); a 27 % reduction in MDA, $p < 0.001$, a 10 % catalase activation, $p < 0.01$; succinate dehydrogenase normalization, and a decrease in dystrophic changes in the articular system of animals.
Conclusion. The results of hematological, biochemical and radiological tests prove that pathological biochemical and morphological changes related to administration of inactivated *M. tuberculosis* to warm-blooded animals can be modified by a mixture of citric and succinic acids added to feed, which allows a better understanding of the pathogenesis and an increased therapy effectiveness.
Keywords: mitochondrial activity, inflammatory process, citric acid, mycobacteria, connective tissue disorders, lipid peroxidation, infection resistance, tuberculosis sanogenesis, tissue respiration, succinic acid.

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Author contributions: *Skupnevskiy S.V*. developed the concept and study design; *Trukhina G.M.* described the mechanisms of toxic effects of *Mycobacterium tuberculosis*; *Pukhaeva E.G.*, *Badtiev A.K.*, *Rurua F.K.*, and *Batagova F.E.* conducted the experiment and estimated hematological and biochemical parameters; *Farnieva Zh.G.* did statistical data processing; all authors contributed to the literature review, read and approved the final version of the article before publication.
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Introduction. According to the U.S. Centers for Disease Control and Prevention, 1.7 billion people were infected with Koch's bacillus in 2018, which is about 23 % of the world population[1]; 5–10 % of the infected usually develop tuberculosis (TB) [1]. The same year, the high-level meeting of UN General Assembly on the fight against tuberculosis adopted the declaration titled "United to End Tuberculosis: An Urgent Global Response to a Global Epidemic" aiming to reduce TB incidence rate by 2030 by 80 % compared to 2015. The annual Global Tuberculosis Report[2] notes that "...*urgent and more ambitious investments and actions are required to put the world on track to reach targets, especially in the context of the coronavirus disease (COVID-19) pandemic*." Despite all the effective measures taken to reduce the spread of infectious *M. tuberculosis*, the disease is among the top 10 global causes of death.

Recent studies have shown that many immunological aspects in the pathogenesis of tuberculosis remain unknown. Some authors [2–6] note that the main reasons for a wide variability of clinical manifestations of the disease are specific features of interaction between the infectious agent and the immune system of the host. In addition to the primary barrier formed by epithelial cells of the respiratory airways, mainly by secretion of IgA immunoglobulin, phagocytic cells of the innate immune system help fight the infection [7–9]. The role of neutrophils and macrophages, however, is dual because under certain conditions not only do they contribute to elimination of tuberculosis bacteria, but also provide a depot (or incubator) for the pathogen [10].

Epidemiological studies have demonstrated that close contacts of x-ray positive pulmonary TB cases with healthy people (within a family; during a long-term stay in the same cabin of the ship; at work and/or school) may infect only 5 % to 20 % of the latter [11]. One of the main reasons for the identified immunity is genetic polymorphism [12–14] that resists the infection thanks to genes in the loci on chromosomes 2q21-2q24, 5p13-5q22, and TST1 in the chromosomal band 11p14 [15, 16]. Mendelian susceptibility to mycobacterial disease characterized by predisposition to local or disseminated infections is also determined by genetic factors leading to disruption of the interleukin/interferon (IL12/IFNγ)-dependent signaling pathway guiding differentiation of lymphocytes by the Th1 type [17]. Population studies indicate close correlations between individual genes and susceptibility

[1] Tuberculosis. https://www.cdc.gov/globalhealth/newsroom/topics/tb/index.html
[2] Global Tuberculosis Report 2020. Geneva: World Health Organization, 2020.

to tuberculosis [18–20]. Aravindan PP [21] provides a detailed review of genes known to determine high susceptibility of humans to *M. tuberculosis* such as the monocyte chemoattractant protein-1 (MCP-1), natural resistance-associated macrophage protein 1 (NRAMP1), toll-like receptors (TLR,) interleukin (IL-8), etc. Findings of scientific studies on the influence of external and internal (mostly genetic) factors on lability of the organism to tuberculosis mycobacteria help identify populations at risk and take targeted preventive actions [22–24]. Comprehensive prevention of complications of both acute and chronic tuberculosis, such as systemic and metabolic dysfunctions, structural pathologies of organs and tissues [25–27], may also lower related disability and mortality rates. A laboratory experiment on warm-blooded animals is widely used to study the etiology and pathogenesis of tuberculosis complications. Complete Freund's adjuvant (CAF, a mineral oil emulsion containing heat-killed *Mycobacterium tuberculosis*) finds application among the model agents that cause systemic pathologies following the effect of lipopolysaccharides of *M. tuberculosis* on the organisms [28]. Its administration to experimental animals induces generalized inflammation and a wide range of autoimmune diseases such as rheumatoid arthritis, encephalomyelitis, uveitis, kidney damage, etc. [29–32]. The use of the model of adjuvant-induced autoimmune disorders also contributes to the search for safe and effective prophylactic medications [33–36].

Our objective was to study the role of citric and succinic acids in protective and adaptive processes in warm-blooded animals with connective tissue disorders induced by inactivated mycobacteria.

Materials and methods. The studies were carried out on male *Wistar* rats ($n = 29$, 400–440 g body weight) aged 24 months, purchased from the *Rappolovo* Laboratory Animal Breeding Facility, Leningrad Region. The rats were kept under standard vivarium conditions at constant temperature, humidity, 12/12 h light/dark cycle, and with free access to feed and water. All researchers complied with the requirements of the Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research (National Research Council Guidelines, 2011; https://www.apa.org/science/leadership/care/care-animal-guidelines.pdf) and GOST R 53434-2009, *Principles of Good Laboratory Practice*. The study was approved by the Ethics Committee at the Institute of Biomedical Research of the Russian Academy of Sciences, Vladikavkaz, Russian Federation. The rats were euthanized in a CO_2 chamber. All solutions were injected under light general anesthesia with Zoletil™ (Virbac, Carros, France). The rodents were divided into four groups. The first group (negative controls, $n = 6$) were injected subcutaneously with the isotonic sodium chloride solution in a volume of 0.1 mL per 200 g body weight (BW) into the right hind limb. The second group (positive controls, $n = 7$) was injected once with complete Freund's adjuvant (CAF) produced by Difco Laboratories Ltd. (Detroit, Michigan, USA) in a volume of 0.1 mL per 200 g BW into the right hind limb. Animals of the third and fourth groups (8 animals each) with disorders induced by injection of 0.1 mL CAF per 200 g BW received once a day with feed a mixture of citric and succinic acids (CA and SA, respectively), in a ratio of 1 : 4 by weight, for 4 weeks. The dosage of acids (neutralized to pH = 7.5 with mineral water) was 17 mg/kg and 88 mg/kg BW in the third and fourth groups, respectively. The entire experiment lasted 7 weeks.

Hematological, biochemical, and cytobiochemical tests were performed using whole blood of animals sampled from the heart under general anesthesia with Zoletil™.

The number of blood leukocytes (WBCs) was counted using an automated hematology analyzer Mythic 22 (Poland).

The activity of succinate dehydrogenase (SDH) in blood lymphocytes was determined by the cytobiochemical method proposed by M.N. Kondrashova and co-authors[3]. For this purpose, 10 μL of blood was dropped onto glass slides, and smears were prepared using a Microscopy Vision device (Austria). The microscope slides were dried and fixed in a 60 % acetone solution for 30 s and then rinsed with distilled water. SDH activity was determined as the difference in indicators (No. 1 – No. 2) of the color intensity of granules obtained by incubating blood cells for 1 hour at $t = 37$ °C in Solution 1 consisting of 125 mmol/L KCl, 10 mmol/L HEPES, 1 mg/mL oxygenated nitroblue tetrazolium (NBT), and 5 mmol/L succinic acid; and Solution 2 containing 125 mmol/L KCl, 10 mmol/L HEPES, 1 mg/mL NBT, and 5 mmol/L malonic acid used as a selective inhibitor of SDH (pH 7.2 ± 0.01). After incubation, the slides were washed with distilled water, dried, and stained in a 0.05 % neutral red solution, which has an affinity for cell nuclei. The preparations were analyzed by light microscopy at 1,000× magnification under oil immersion. For each rat, we examined 100 cells randomly chosen from three microscope slide regions: anterior, middle, and posterior. The preparations were photographed and processed using the Bio Images software (Pushchino, Russian Federation), which allows calculating the area and color intensity of diformazan granules serving as markers of mitochondrial respiratory activity.

For biochemical testing, blood was centrifuged and fractionated. Hydroperoxides in plasma were determined according to Gavrilov using test kits by Agat-Med LLC (Russian Federation), and the level of malondialdehyde was measured by the thiobarbituric acid reactive substance assay in erythrocytes preliminarily washed with a cold ($t = 4$ °C) isotonic solution[4]. Catalase activity was studied using a method based on spectrophotometric measurement of hydrogen peroxide decomposition rate at a wavelength of 230 nm (T. Beutler[5]).

X-rays were obtained on a stationary veterinary X-ray machine Ecoray Ultra 300V (Seoul, South Korea).

We estimated the mean and its standard error. The normal distribution of the data was checked using the Shapiro–Wilk test and, in case of positive conclusion, the hypotheses were compared using the Student's *t*-test in Microsoft Excel. Differences were considered statistically significant at $p \leq 0.05$. Correlation coefficients (*rxy*) were calculated according to Pearson.

Results. The mycobacterial component of complete Freund's adjuvant provides in the long term (for small

[3] Kondrashova MN, Zakharchenko MV, Khunderyakova NV, Maevskiy EI. [Cytobiochemical method for determining the activity of succinate dehydrogenase, the oxidation of endogenous succinic acid, the signaling effect of micromolar concentrations of succinic acid, its use for quantifying the level of adrenergic regulation in the body, the medium and the kit for implementing the method.] Patent RU 2364868 C1. (In Russ.)

[4] [*Methods of Clinical Laboratory Testing.*] Kamyshnikov VS, ed. 8th ed. Moscow: MEDpress-inform Publ., 2016. (In Russ.)

[5] [*Handbook of Laboratory Research Methods.*] Danilova LA, ed. Moscow: Piter Publ., 2003. (In Russ.)

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laboratory animals – within several weeks) remodeling of the hematopoietic system and release of immature leukocytes (myelopoietic cell) into the circulation [37], which is confirmed by the results presented in Fig. 1.

The bar graph shows that the immune response to the injection of *M. tuberculosis* antigens was manifested by leukocytosis since the number of WBCs in model animals increased by 28 % compared to the controls ($p = 0.016$), thus indicating chronic inflammation. Owing to administration of a mixture of organic acids with antioxidant activity, the severity of responses differed qualitatively depending on the dose. The minimal dose of 17 mg/kg BW made the immunological response 15 % greater than that in the positive control group, while the maximal dose of 88 mg/kg BW helped maintain the hematological indicator within the normal range (comparable to that of the negative control) differing significantly from the indicators of the model group by 22 % ($p = 0.039$).

The important role of free radical oxidation processes in the mechanism of inflammation determines the interest to assessing markers of lipid peroxidation in blood of experimental animals (Fig. 2).

The figure shows that antigenic stimulation of the body is accompanied by disturbance of redox homeostasis with an increase in pro-oxidant processes. The scattering of indicators of the primary lipid peroxidation products (hydroperoxides) between the norm and pathology is 17 % (insignificant) and for secondary products including malondialdehyde (thiobarbiturates) – already 40 % ($p < 0.001$). The therapeutic effect of the citrate-succinate mixture leads to a dose-dependent normalization of processes involving such highly reactive intermediates as free oxygen radicals, which is manifested by a statistically significant decrease in MDA by 3 % (minimum dosage) and 27 % (maximum dosage, $p < 0.001$).

The protective effect of organic acids on antioxidant enzymes is shown in Fig. 3.

Administration of CAF to animals leads to a slight (by 4 %) inactivation of catalase, one of the key enzymes responsible for the inactivation of reactive oxygen species. Consumed citric and succinic acids contribute to statistically significant reactivation of H_2O_2-oxidoreductase by 10 % ($p = 0.01$) and 5 % in groups with minimum and maximum dosages, respectively.

Figure 4 shows the functional state of mitochondria in immunocompetent cells by the key respiratory enzyme SDH.

According to our findings, one of the manifestations of immunotoxic effect of CAF is an imbalance in cellular respiration. In animals with the modelled pathology, SDH is activated by 23–39 % (insignificant); at this, the administration of the Krebs cycle substrates (citric and succinic acids) in the minimum dosage does not affect the state of the second respiratory complex against the developed disorder while the

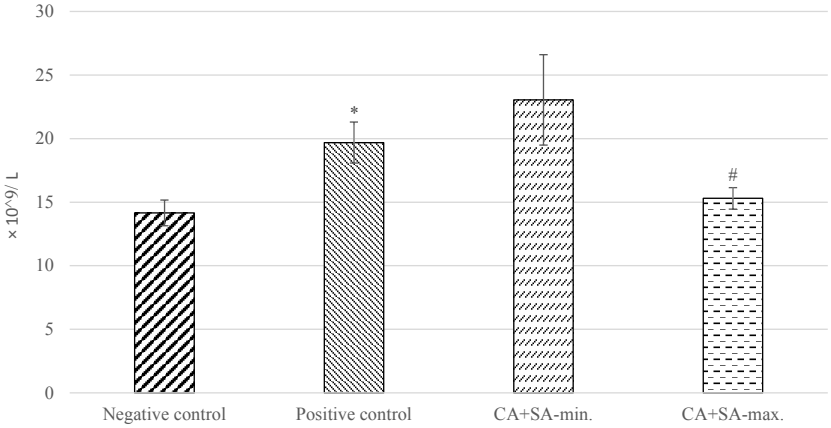

Рис. 1. Общее количество лейкоцитов в крови лабораторных животных
(* $p < 0,05$ относительно негативного контроля, # $p < 0,05$ относительно позитивного контроля)
Fig. 1. White blood cell counts in the rats (* $p < 0.05$ compared to the negative control, # $p < 0.05$ compared to the positive control)

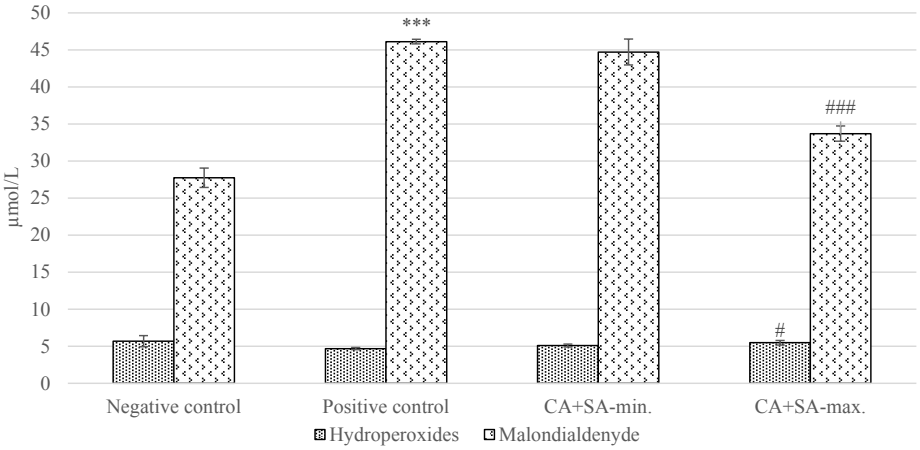

Рис. 2. Содержание продуктов липопероксидации в крови у крыс (*** $p < 0,001$ относительно негативного контроля, # $p < 0,05$, ### $p < 0,001$ относительно позитивного контроля)
Fig. 2. Concentration of lipoperoxidation products in the blood of rats (*** $p < 0.001$ compare to the negative control, # $p < 0.05$, ### $p < 0.001$ compare to the positive control)

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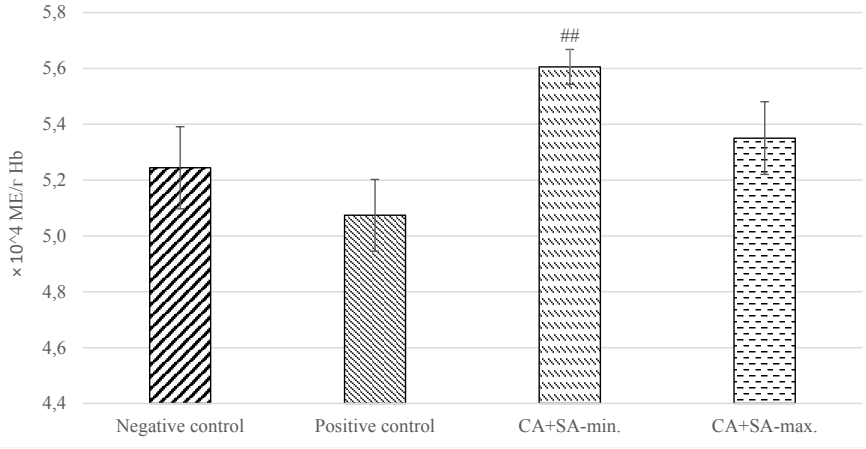

Рис. 3. Активность эритроцитарной каталазы у крыс
Fig. 3. Activity of erythrocyte catalase in the rats

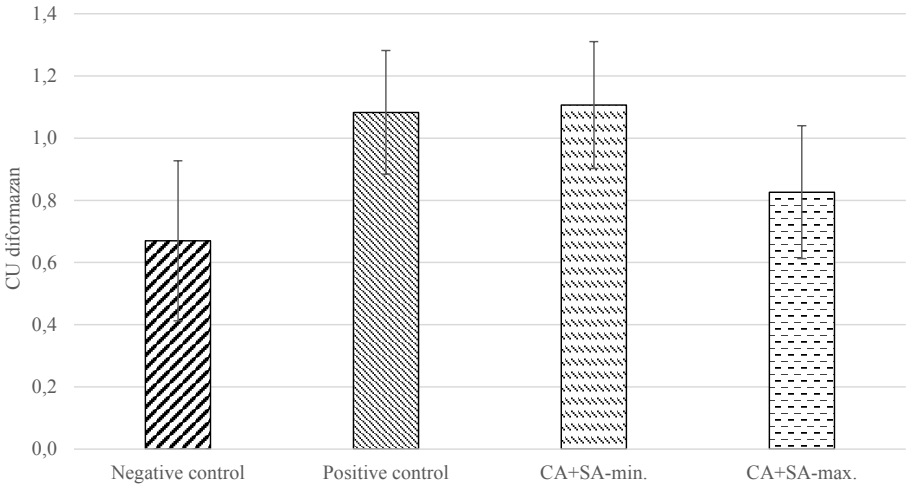

Рис. 4. Активность сукцинатдегидрогеназы в митохондриях лимфоцитов у крыс
Fig. 4. Activity of succinate dehydrogenase in the mitochondria of lymphocytes in rats

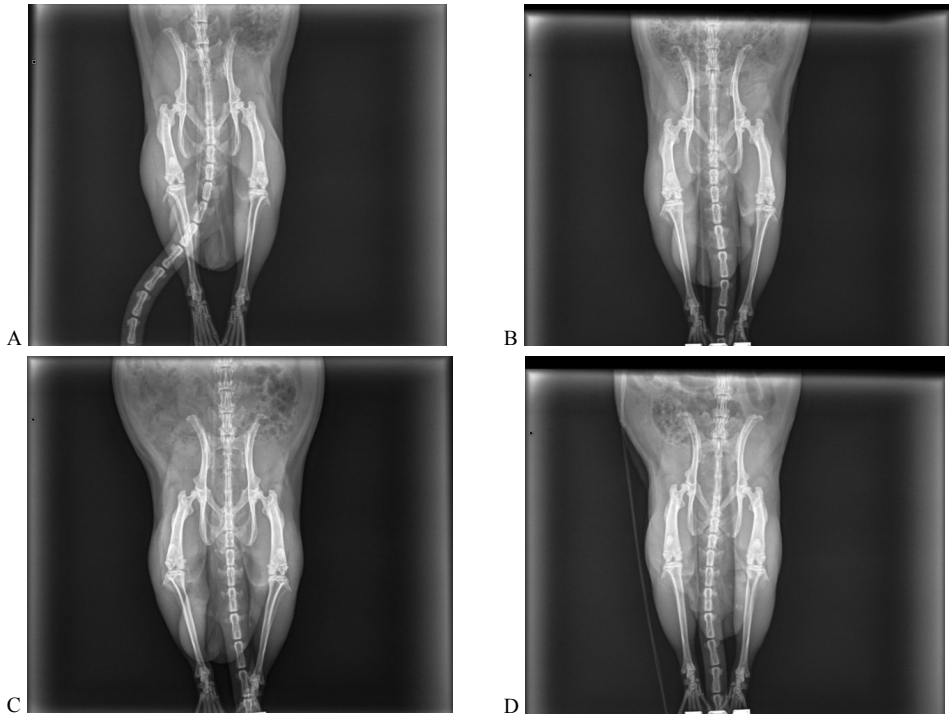

Рис. 5. Ренгенологические снимки экспериментальных животных (A – негативный контроль; B – позитивный контроль; C, D – животные, получавшие смесь лимонной и янтарной кислот в минимальной и максимальной дозах)
Fig. 5. X-ray images of experimental animals (A – negative control; B – positive control; C and D – animals given a mixture of citric and succinic acids in minimum and maximum doses)

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maximum dosage promotes normalization of oxidative phosphorylation.

Taking into account the involvement of connective tissue in the systemic inflammatory response syndrome induced by the injected oil emulsion of heat-killed *M. tuberculosis* in the animals, the study of the musculoskeletal system enables evaluation of therapeutic effectiveness (Fig. 5).

The articular system of the animals in the model group is characterized by cyst-like white spots in the heads of bones and their subchondral sclerosis. Uneven narrowing of joint spaces is typical of these animals. The warm-blooded animals that received antioxidant therapy in the diet are characterized by a decrease in dystrophic changes manifested by uneven narrowing of joint spaces and small cyst-like white spots; yet, the described changes can be reversible.

The animals of the negative control group had no visible pathological lesions.

Discussion. As of today, the increased risks of tuberculosis and opportunistic infections are associated with inclusion of anti-cytokine drugs in the pathogenetic therapy for COVID-19[6]. Measures taken to enhance the resistance of the organism and prevent complications of the underlying condition (i.e. tuberculosis) should contribute to reduction of the collateral damage of their wide use [38, 39]. Maintaining the redox balance during the inflammatory process (especially that of the infectious etiology) is aimed at preventing oxidative stress and preserving high barrier properties of membranes impeding cell contamination. In this connection the antioxidant effect of a mixture of citric and succinic acids manifested by a dose-dependent decrease in the blood level of malondialdehyde in the experimental animals with *M. tuberculosis*-induced disorders increases the resistance of the body to the pathogen. This effect is based not only on the direct action of natural antioxidants inhibiting the oxidative process, but also on their stimulation of enzymes of the antioxidant defense system, as shown by catalase activation in treated animals (Fig. 3). The established high inverse correlation between the activity of SDH and level of hydroperoxides in plasma $rxy = -0.90$ also substantiates the regulatory role of organic acids in redox homeostasis: against the background of activation of cell respiration, stabilization of the release of primary products of oxidative destruction in membranes is noted. Evidence of an increase in the body's resistance to damage through citric and succinic acids is the results of X-ray studies, which indicate a modification of the pathogenesis of autoimmune rheumatoid arthritis and the possibility of arresting pathomorphological changes even in the late stages of the disease.

Conclusion

A mixture of citric and succinic acids contributes to an increase in immune resistance in warm-blooded animals with *M. tuberculosis*-induced disorders, which is manifested by a reduced inflammation, suppression of oxidative stress, including through activation of antioxidant enzymes, normalization of cellular respiration, and enhancement of cellular barriers. Correction of the immune status with a mixture of organic acids lowered the risk of developing musculoskeletal disorders (autoimmune rheumatoid arthritis) caused by the effect of *M. tuberculosis* antigens on the body.

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