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Nonclinical study of the new immunotropic drug effectiveness in salmonella infection treatment

Teymurazov M.G.[1], Petrova N.V.[2, 3], Karelina E.A.[3], Ganina K.K.[3], Tarasov S.A.[2, 3], Epstein O.I.[2, 3]

[1] State Research Center for Applied Microbiology and Biotechnology (SRCAMB)
24, Kvartal A, Obolensk, Moscow Region, 142279, Russian Federation

[2] Institute of General Pathology and Pathophysiology (IGPP)
8, Baltiyskaya Str., Moscow, 125315, Russian Federation

[3] MATERIA MEDICA HOLDING LLC, Research and Production Company
47/1, Trifonovskaya Str., Moscow, 129272, Russian Federation

ABSTRACT

The aim of the study was to evaluate the immunoregulatory activity of the experimental drug based on ultra-high dilutions of antibodies to MHC I and MHC II molecules against *Salmonella enteritidis rif92*.

Materials and methods. The drug tested: a sample of ultra-high water-alcohol dilutions of antibodies to MHC I and MHC II molecules applied to lactose powder (the theoretical level of the initial antibody concentration reduction is at least 10^{24} times). A model of non-lethal salmonella infection in chickens was induced by administering a virulent strain of *Salmonella enteritidis rif92* with a concentration of 2.5×10^9 CFU / g in the volume of 0.5 ml / bird. The following groups were formed (n = 15 in each group): 1 – drug; 2 – drug + antibiotic at the median effective dose (ED 50); 3 – placebo; 4 – placebo + antibiotic at ED50; 5 – intact control. The duration of the experiment was 12 days. The studied parameters included the survival rate during the observation period; daily body weight; feed consumption for the entire period; pathogen concentration in the litter on day 3, 6, and 9; the presence and concentration of the pathogen in the liver and cecum on day 12; and the index of antimicrobial activity on day 12.

Results. In the groups receiving the experimental drug, the infectious process proceeded in a milder form and the bacterial load in chickens was lower. The bacterial count in the litter was reduced by two orders compared to the respective control when the drug was added both alone and in combination with the antibiotic. A protective effect of the experimental drug on the liver of the infected chickens was detected.

Conclusion. A pronounced immunoregulatory activity of the studied drug against *Salmonella enteritidis rif92* in chickens was demonstrated for the first time. The results obtained allow to consider the drug as a promising agent for the treatment of salmonella infection.

Key words: salmonellosis, ultra-high dilutions of antibodies, MHC class I and II molecules, antibiotics, chickens.

Conflict of interests. N.V. Petrova, E.A. Karelina, K.K. Ganina, and S.A. Tarasov are employees of MATERIA MEDICA HOLDING LLC. O.I. Epstein is the founder and the President of MATERIA MEDICA HOLDING LLC. The decision to publish the results of the study belongs to MATERIA MEDICA HOLDING LLC. Patent applications for the substances and the drug have been submitted by MATERIA MEDICA HOLDING LLC and O.I. Epstein.

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Conformity with the principles of ethics. The study protocol was approved by the Ethics Committee of State Research Center for Applied Microbiology and Biotechnology (Protocol No. of 03.12.2018).

✉ *Petrova N. V.,* email: physactive@yandex.ru.

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Доклиническое изучение эффективности нового иммунотропного препарата при лечении сальмонеллезной инфекции

Теймуразов М.Г.[1], Петрова Н.В.[2, 3], Карелина Е.А.[3], Ганина К.К.[3], Тарасов С.А.[2, 3], Эпштейн О.И.[2, 3]

[1] Государственный научный центр прикладной микробиологии и биотехнологии (ГНЦ ПМБ)
Россия, 142279, Московская обл., Серпуховский р-н, пос. Оболенск, территория «Квартал А», 24

[2] Научно-исследовательский институт общей патологии и патофизиологии (НИИОПП)
Россия, 125315, г. Москва, ул. Балтийская, 8

[3]ООО «НПФ «Материа медика холдинг»
Россия, 129272, г. Москва, ул. Трифоновская, 47 стр. 1

РЕЗЮМЕ

Цель исследования – оценить иммунорегуляторную активность экспериментального препарата на основе сверхвысоких разведений антител к молекулам МНС I и II в отношении *Salmonella enteritidis rif92*.

Материалы и методы. Изучаемый препарат: образец сверхвысоких водно-спиртовых разведений антител к молекулам МНС I и II, нанесенных на порошок лактозы (теоретический уровень снижения концентрации исходных антител как минимум в 10^{24} раз). Модель – нелетальная сальмонеллезная инфекция у цыплят. Заражение проводили вирулентным штаммом *S. enteritidis rif92* концентрацией 2,5 × 10^9 КОЕ/г в объеме 0,5 мл/голову. Группы (n = 15 в каждой): 1 – препарат; 2 − препарат + антибиотик в дозировке 50%-й эффективной дозы (ЭД50); 3 − плацебо; 4 − плацебо + антибиотик в дозировке ЭД50; 5 − интактный контроль. Продолжительность эксперимента 12 сут. Изучаемые показатели: выживаемость в течение периода наблюдения, масса тела ежедневно, затраты корма за весь период, концентрация возбудителя в помете на 3, 6, 9-е сут, наличие и концентрация возбудителя в печени и слепых отростках тонкого кишечника, а также индекс антимикробной активности на 12-е сут.

Результаты. В группах с введением экспериментального препарата инфекционный процесс проходил в более легкой форме, бактериальная нагрузка у цыплят была ниже. Обсемененность помета снижалась на два порядка по сравнению с соответствующим контролем при добавлении препарата как в виде монотерапии, так и в сочетании с антибиотиком. Выявлено протективное действие препарата на печень зараженных цыплят. Заключение**.** Впервые продемонстрирована выраженная иммунорегуляторная активность изучаемого препарата в отношении *Salmonella enteritidis rif92* у цыплят. Полученные результаты позволяют рассматривать данный препарат в качестве перспективного агента для терапии сальмонеллезной инфекции.

Ключевые слова: сальмонеллез, сверхвысокие разведения антител, молекулы МНС I и II классов, антибиотики, цыплята.

Конфликт интересов. Петрова Н.В., Карелина Е.А., Ганина К.К. и Тарасов С.А. – сотрудники компании ООО «НПФ «МАТЕРИА МЕДИКА ХОЛДИНГ». О.И. Эпштейн – основатель и Президент компании ООО «НПФ «МАТЕРИА МЕДИКА ХОЛДИНГ». Решение о публикации результатов научной работы принадлежит ООО «НПФ «МАТЕРИА МЕДИКА ХОЛДИНГ». Заявителями на получение патента на указанные субстанции и препарат являются ООО «НПФ «МАТЕРИА МЕДИКА ХОЛДИНГ» и О.И. Эпштейн.

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INTRODUCTION

Salmonella infection tends to result from consumption of low-quality food, primarily eggs and poultry meat [1, 2–5]. The infectious process usually proceeds without complications, but a severe form can be observed in patients with impaired immune status, as well as in children and elderly people [1, 6–7]. If the disease has a mild or moderate course, antimicrobial therapy is not recommended in healthy people [8], since most antimicrobial drugs are active against salmonella only during the incubation period and at the beginning of the disease [9]. In addition, excessive use of antimicrobials contributes to the development of pathogen resistance to drugs through multiple molecular and genetic mechanisms [1, 8, 10–12]. Thus, the use of drugs that affect the targets expressed by immune cells can be considered as a relevant and promising direction in treatment of salmonellosis [13–17].

We conducted an *in vivo* study using a model of non-lethal salmonella infection in chickens, which was aimed at assessing the activity of a new drug based on ultra-high dilutions of antibodies to MHC class I and II molecules. The drug was developed by MATERIA MEDICA HOLDING LLC and has a modulatory effect aimed at the targets of immune cells.

MATERIALS AND METHODS

The experimental drug was a lactose powder saturated with ultra-high dilutions of antibodies to MHC class I and II molecules and obtained using the following technology: affinity-purified rabbit polyclonal antibodies to MHC I and MHC II were used as the initial substance and subsequently utilized for the preparation of ultra-high dilutions. To obtain a 100-fold dilution, the substances were diluted in an aqueous-alcohol solution in the ratio of 1 : 100 with vigorous stirring. The final dilutions of antibodies to MHC I and MHC II contained a mixture of the 12^{th}, 30^{th} and 50^{th} centesimal dilutions.

Thus, if the identified special physical and chemical features typical of highly diluted substances [18–20] are not taken into account, the theoretical level of reduction in the concentration of the initial antibodies can be 10^{24} times. Lactose monohydrate was saturated with the resulting dilutions using the fluidized bed unit. Lactose powder with an aqueous alcohol solution applied on it, which was obtained with a similar technology of ultra-high dilutions in purified water, was used as a placebo. The samples of the drug and placebo were supplied and tested blinded. Unblinding was carried out after the end of the experiment and statistical analysis of the data obtained.

For the study, 2.5% aqueous solutions of the drug and placebo were prepared and administered orally to chickens once a day at a dose of 0.2 ml per bird. Ciprofloxacin hydrochloride was used as an antibacterial drug. In preliminary studies, its efficacy against the pathogen was confirmed and a median effective dose (ED50) was calculated. Inoculation was carried out using a virulent *Salmonella enteritidis rif92* strain obtained from the State Collection of Pathogenic Microorganisms and Cell Cultures (SCPM-Obolensk) with a non-lethal concentration of 2.5×10^9 CFU / g in the volume of 0.5 ml / bird.

Five groups of 15-day-old cross Cobb broiler chickens obtained from Novo-Petrovskaya Poultry Farm LLC were used in the study. For *in vivo* models of salmonellosis, rodents are usually proposed [21], but the used strain of *Salmonella enteritidis* is associated with poultry and poultry products, which are a source of human infection [4], and it is one of the main pathogens of food toxicoinfection in humans [22]. Infections in mice develop in the absence of pronounced symptoms of diarrhea, so the rodent model may not be sufficiently informative [23].

We employed methods that allowed to assess the bacterial load of internal organs in order to quantify virulence: the gastrointestinal tract of chickens is an optimal system for studying intestinal zooanthroponotic infections [24]. Taking into account the intended treatment, the following groups of chickens were formed in the experiment: 1 – drug; 2 – drug + antibiotic; 3 – placebo; 4 – placebo + antibiotic; 5 – intact control. All groups were under the same housing conditions. The duration of the study was 12 days (from day 1 to day 12 of the chickens' life).

The chickens were quarantined for the first two days. On day 3, they were randomized into groups and infected with *Salmonella enteritidis rif92* (except for the intact group). On days 4–9 of life, the studied drug or placebo was administered to chickens of groups 1–4. In addition, on days 5–9 of life, the chickens of the 2^{nd} and 4^{th} groups received ciprofloxacin hydrochloride orally at ED50 (0.5 mg / kg body weight) in the volume of 0.2 ml. The experimental drug or placebo, respectively, and the antibiotic were administered at one-hour intervals. The following parameters were assessed: the survival rate during the experiment; daily body weight; feed consumption per 1 kg of weight gain over the entire observation peri-

od; the concentration of *Salmonella enteritidis rif92* in the litter on days 3, 6, 9 of the experiment; the presence and concentration of the pathogen in the liver and cecum on day 12, and the index of antimicrobial activity (IAA).

Colonization of the intestine by *Salmonella enteritidis rif92* was monitored by bacteriological analysis of the feces of the infected chickens. On days 3, 6, and 9, the pool of feces of the entire group was studied; on day 12, it was studied individually after euthanasia. The persistence level of *Salmonella enteritidis rif92* was estimated according to the number of bacteria in one gram of the litter. The presence and concentration of *Salmonella enteritidis rif92* in the intestine were determined according to ISO 6887-1983 *General Guidance for the Preparation of Dilutions for Microbiological Examination*.

The colonies grown on the nutrient media were counted and identified using salmonella diagnostic sera during mass spectrometry analysis.

IAA was calculated as a ratio of the microbial cells contained in the organ homogenate in the control group to those in the experimental group at the end of the observation period [25].

Statistical analysis was performed using the Microsoft R Open 3.4.4 platform. Based on the primary survival rate data, a statistical model was developed for comparing the groups using the log-rank test. The Holm – Bonferroni method was used for multiplicity adjustment. According to the body mass index, the arithmetic mean and the standard error of the mean were calculated for each group. The groups were compared using two-factor linear models and post-hoc Tukey's honestly significant difference (HSD) test. To analyze the concentration of the pathogen in the liver and intestinal contents of the infected chickens, the arithmetic mean and standard error of the mean were calculated. The groups were compared using the Kruskal – Wallis test and the Wilcoxon signed-rank test. The differences between the groups were considered statistically significant at $p < 0.05$.

RESULTS

The survival rate of chickens in all the groups was 100%. The average values of the chickens' body weight from day 1 to day 9 of the study were comparable in all the groups. Starting from day 10, the body weight in the drug group was higher than in the other groups. At the same time, statistically significant differences were observed: on day 10, the drug group as opposed to the placebo group (260.0 ± 72 g vs. 228.8 ± 8.7 g); on day 11 – as opposed to the intact group (297.0 ± 8.4 g vs. 263.0 ± 15.8 g); on day 12 – as opposed to the placebo group (325.8 ± 9.6 g vs. 291.9 ± 10.2 g) and the intact control (325.8 ± 9.6 g vs. 287.1 ± 16.2 g). The remaining groups were comparable in terms of body weight.

The best values for feed consumption per 1 kg of body weight gain were obtained in the drug group (1.28 kg), in the drug + antibiotic group (1.29 kg), and in the placebo + antibiotic group (1.27 kg), while this value was 1.39 kg in the placebo group, and 1.50 kg in the intact control group.

The dynamics of *Salmonella enteritidis rif 9* concentration in chickens' litter is given in Table 1.

Table 1

***Salmonella enteritidis* concentration per gram of fecal pool post infection (CFU / g)**

Groups	Day 3	Day 6	Day 9
Drug, $n = 15$	5×10^6	2×10^2	74
Drug + antibiotic, $n = 15$	5×10^6	1×10^2	32
Placebo, $n = 15$	7×10^6	6×10^4	4×10^2
Placebo + antibiotic, $n = 15$	5×10^6	5×10^4	39
Intact control, $n = 15$	-	-	-

On day 6 post infection, in the groups where the experimental drug was administered both alone and in combination with the antibiotic, the salmonella concentration was reduced by 4 orders of magnitude, while in the placebo + antibiotic group – only by 2 orders of magnitude. On day 9, the presence of *Salmonella enteritidis* in the chickens' litter remained only in the placebo group. In the other groups, the concentration of the pathogen was represented by single colonies.

The data on the number of infected chickens, the bacterial count of *Salmonella enteritidis* in the liver, and the presence of the pathogen in the intestinal contents for all the groups at the end of the experiment (on day 12 post infection) are given in Table 2.

On day 12 post infection, the pathogen in the intestine was observed in almost half of the chickens in the placebo group. The addition of the antibiotic reduced the percentage of infected chickens, but the best results were obtained in the drug and drug + antibiotic groups, and in the latter, the concentration of the pathogen was minimal. As for the pathogen detected in the liver, low percentage of invasion was observed in all the groups, but the best results were obtained in the drug + antibiotic group.

Table 2

The presence of *Salmonella enteritidis* in the infected chickens after euthanasia and the index of antimicrobial activity

Groups	Presence of *S. enteritidis* in the liver		Index of antimicrobial activity	Presence of *S. enteritidis* in the intestinal contents		Index of antimicrobial activity
	n (%)	Concentration, CFU/g ($M \pm SE$)		*n* (%)	Concentration, CFU/g ($M \pm SE$)	
Drug	2 (13.3)	18.5 ± 14.5	10.6	2 (13.3)	$(5.5 \pm 2.5) \times 10^3$	0.4
Drug + antibiotic	1 (6.7)	56.0 ± 0	3.6	4 (26.7)	$(5.0 \pm 1.2) \times 10^2$	4.6
Placebo	3 (20.0)	195.7 ± 52.3	-	7 (46.7)	$(2.3 \pm 6.3) \times 10^2$	-
Placebo + antibiotic	1 (6.7)	200.0 ± 0	0.9	5 (33.3)	$(3.8 \pm 1.7) \times 10^3$	0.6
Intact control	–	–	–	–	–	–

The antimicrobial activity of the antibiotic administered at ED50 was low (the placebo + antibiotic group). However, the addition of the drug increased IAA by 4.0 and 7.7 times in the liver and intestine, respectively. We also observed high IAA of the experimental drug during liver examination, which indicates its protective effect in case of salmonella invasion.

DISCUSSION

The targets of the experimental drug examined in this study are MHC class I and class II molecules. Based on the previously shown properties of this drug class [26, 27], the experimental drug obviously influences its targets by activating the processing and presentation of the antigen and forming an adequate immune response during the infectious process. MHC class I molecules present peptide antigenic determinants to naive CD8+ killer T cells, while MHC class II molecules – to naive CD4+ T helper cells and regulatory T cells [28]. The MHC system molecules are now considered as some of the most promising markers of specific adaptive immune responses, including antigen processing and presentation. The mechanisms of their functioning are investigated in the studies on infectious diseases [29–32].

In this study, experimental infection of chickens with a reduced dose of *Salmonella enteritidis rif92* resulted in the development of a non-lethal infection, which is manifested by prolonged (in some chickens, until the last day of the study) presence of the pathogen in the gastrointestinal tract. The difference between the groups in terms of body weight is worth noting as an indicator of the overall condition. In the chickens treated with the experimental drug, it was significantly higher than in the placebo and intact control groups as well as in the antibiotic groups. This indirectly confirms the development of milder infectious process in the group that received the experimental drug.

The best results in terms of feed consumption per 1 kg of weight gain were observed in the groups where chickens were treated with the experimental drug or the antibiotic, as well as a combination of them. The pathogenesis of this model is associated with a violation of the morphological and functional characteristics of the gastrointestinal tract, so a decrease in feed consumption indicates a higher ability of the intestine to digest and absorb nutrients and, consequently, a lower negative impact of infection. The effectiveness of treating chickens with the experimental drug in terms of nutrient absorption was comparable to that of antibiotic therapy or a combination of them.

The therapy with the experimental drug reduced the time of chickens' recovery. Thus, day 6 post infection was characterized by a biologically significant decrease in the concentration of the pathogen in the litter by two orders of magnitude in the groups receiving the experimental drug both alone and in combination with a reduced dose of the antibiotic compared to the groups receiving placebo.

Adding the experimental drug to the antibiotic increased the level of antimicrobial activity of the latter by 4 and 7.7 times in the liver and intestine, respectively. At the same time, the intrinsic antimicrobial activity of the experimental drug in the liver was high. The decrease in salmonella invasion in the liver may be associated with the protective effect of the drug on the walls of the gastrointestinal tract, which prevented the penetration of the pathogen into the bloodstream and internal organs. The results of studies by other authors [13–15, 33] also confirm the assumption about immune mechanisms in the defense against salmonella.

CONCLUSION

Pronounced immunoregulatory activity of the studied drug against *Salmonella enteritidis rif92* was shown for the first time. The results obtained allow to

consider this drug as a promising agent for treatment of salmonellosis both alone and in combination with an antibiotic.

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Authors contribution

Teimurazov M.G. – planning and carrying out of the laboratory research, analysis and interpretation of the data. Petrova N.V. – planning of the research design, analysis of the results. Karelina E.A.– planning of the research design, analysis of the results, drafting of the article. Ganina K.K. – statistical processing of the results. Tarasov S.A. – management of the project. Epstein O.I. – development of the drug concept, comments on the manuscript.

Authors information

Teymurazov Marat G., Cand. Sci. (Biology), Senior Researcher, Department of molecular Microbiology, SRCAMB, Obolensk, Russian Federation. ORCID 0000-0002-8635-8366.

Petrova Nataliia V., Researcher, Laboratory of Physiologically Active Substances, IGPP; Senior Researcher, Research and Analytical Division of R&D Department, Materia Medica Holding LLC, Research and Production Company, Moscow, Russian Federation. ORCID 0000-0002-2192-7302.

Karelina Ekaterina A., Cand. Sci. (Vet.), Senior Researcher, Research and Analytical Division of R&D Department, Materia Medica Holding LLC, Research and Production Company, Moscow, Russian Federation. ORCID 0000-0001-6109-4643.

Ganina Ksenia K., Cand. Sci. (Biology), Senior Researcher, Research and Analytical Division of R&D Department, Materia Medica Holding LLC, Research and Production Company, Moscow, Russian Federation. ORCID 0000-0003-1571-6338.

Tarasov Sergey A., Cand. Sci. (Med.), Leading Researcher, Laboratory of Physiologically Active Substances, IGPP; Head of Research and Analytical Division of R&D Department, Materia Medica Holding LLC, Research and Production Company, Moscow, Russian Federation. ORCID 0000-0002-6650-6958.

Epstein Oleg I., Dr. Sci. (Med.) , Corresponding Member of the Russian Academy of Sciences, Head of Laboratory of Physiologically Active Substances, IGPP; President of Materia Medica Holding LLC, Research and Production Company, Moscow, Russian Federation. ORCID 0000-0003-3992-6639.

(✉) **Petrova N. V.,** email: physactive@yandex.ru.

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