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Adjuvant properties of chitosan derivatives administered to mice with anti-rabies vaccine

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ABSTRACT
Searching for a preparation that would meet all the requirements for modern adjuvants remains a matter of critical importance for specific immunoprophylaxis. Much information is available now on chitosan positive effect, including its effect on the immune response. The article provides results of the preclinical tests for different affordable chitosan-based products. For the test purposes, we took the following three products manufactured by LLC Bioprogress (Shchelkovo, Russia): water-soluble chitosan (succinate) – 2% solution edible chitosan (water-soluble) – 2% solution; edible chitosan (acid-soluble) – 2% solution, as well as anti-rabies vaccine RABIKOV manufactured by Shchelkovo Biocombinat (Russia). Immunogenic properties of chitosan-based products were tested in 85–100-day-old female white laboratory mice weighing 21–35 g. The animals were divided into 37 groups (6 mice in each group). Chitosan-based products were administered subcutaneously or intramuscularly, either together with the anti-rabies vaccine or without it. Animals from the control groups received either saline solution or the vaccine only. There was also a group of intact animals. The experiment demonstrated that the water-soluble chitosan (succinate) administered subcutaneously, acid-soluble edible chitosan (at a concentration of 1:64 and more), and water-soluble edible chitosan (at a concentration of $1:10^8$) administered subcutaneously and intramuscularly increase the level of post-vaccination anti-rabies antibodies. Thus, the tested chitosan-based products do not have any negative impact on the laboratory animals and have immunogenic properties.

Keywords: chitosan, adjuvant, vaccination, immunoprophylaxis, RABIES vaccine, RABIKOV vaccine, cytotoxicity, immunity, antibodies, preclinical tests

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Адъювантные свойства производных хитозана при введении мышам антирабической вакцины

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РЕЗЮМЕ
Для специфической иммунопрофилактики особенно актуальным остается вопрос поиска препарата, который бы отвечал всем требованиям, предъявляемым к современным адъювантам. В литературе много сведений о положительном влиянии хитозана, в том числе и на иммунную систему. В статье представлены результаты доклинических испытаний препаратов на основе различных форм хитозана, которые являются экономически доступными. В качестве испытуемых были взяты три препарата производства ООО «Биопрогресс» (г. Щелково, Россия): хитозан водорастворимый (сукцинат) – 2%-й раствор; хитозан пищевой (водорастворимый) – 2%-й раствор; хитозан пищевой (кислоторастворимый) – 2%-й раствор; а также вакцина против бешенства «Рабиков» производства ФКП «Щелковский биокомбинат» (Россия). Изучение иммуногенных свойств препаратов хитозана проводили на 85–100-суточных самках белых лабораторных мышей массой 21–35 г. Животные были поделены на 37 групп по 6 мышей в каждой. Препараты хитозана применяли подкожно или внутримышечно сочетанно с антирабической вакциной или без таковой. Животным контрольных групп вводили либо физиологический раствор, либо только вакцину. Также была сформирована группа интактных животных. Показано, что хитозан водорастворимый (сукцинат) при подкожном введении, хитозан пищевой (кислоторастворимый) в концентрации 1:64 и выше и хитозан пищевой (водорастворимый) в концентрации $1:10^8$ при подкожном и внутримышечном способах введения повышают уровень поствакцинальных антирабических антител. Таким образом, исследуемые препараты на основе хитозана не оказывают негативного влияния на организм лабораторных животных и обладают иммуногенными свойствами.

Ключевые слова: хитозан, адъювант, вакцинация, иммунопрофилактика, вакцина «Рабиес», вакцина «Рабиков», цитотоксичность, иммунитет, антитела, доклиническое исследование

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INTRODUCTION

Scientists from all over the world are looking for the best options to ensure effective and safe specific prevention of animal diseases. Despite the progress made in the vaccine production, searching for a low-cost and safe adjuvant that would enhance the immune response remains a matter of great importance.

Modern adjuvants shall boost immunity (cell-mediated and/or humoral), be easily metabolized and be widely affordable [1, 2].

Chitosan properties are being actively studied worldwide [3–20]. Its derivatives are widely used in the pharmaceutical manufacturing and in veterinary medicine. Chitosan-based products have shown a variety of biological properties, including antimicrobial and cholesterol-lowering ones. The chitosan properties increase the dissociation rate of poorly soluble medicinal products, enhance their absorption, influence the drug release and create drugs of prolonged action [2]. It was also noted that chitosan solution boosts both the humoral and cell-mediated immune response after subcutaneous administration of vaccines [15]. It is also possible to inject chitosan as a part of medicinal products and vaccines [21].

The available literature provides information on chitosan as an antigen sorbent and a stimulator of the post-vaccination immune response [3, 7, 8, 9, 10, 12, 13, 14, 17, 18, 19, 20, 22]. Progress made in studying the properties of chitosan derivatives suggests that they may meet the basic requirements for modern adjuvants.

Previously, in order to find optimal concentrations of chitosan-based products for administration to animals, we analyzed the cytotoxicity of various dilutions of the tested samples in the continuous bovine kidney cell line PT-80 [6, 11].

The purpose of this research is to study adjuvant properties of chitosan derivatives in various concentrations, administered to mice together with an anti-rabies vaccine.

MATERIALS AND METHODS

Chitosan. The following chitosan-containing products manufactured by Bioprogress (Shchelkovo, Russia) on the basis of saline solution (NaCl 0.9%), were taken as starting materials:

– water-soluble chitosan (succinate), 2% solution – Preparation No. 1;

– edible chitosan (water-soluble), 2% solution – Preparation No. 2;

– edible chitosan (acid-soluble), 2% solution – Preparation No. 3.

Anti-rabies vaccines for animals:

– anti-rabies vaccine RABIKOV manufactured by the Shchelkovo Biocombinat (Russia);

– anti-rabies vaccine RABIES (Intervet International, B. V., the Netherlands) was used to compare protective properties with the domestically produced RABIKOV vaccine as part of the implementation of import substitution measures.

Experiment design. Thirty-seven homogeneous groups of female white lab mice were formed for experimental purposes. The mice weighed 21–35 g and were 85–100 days old. Six mice were included in each group. The mice were injected with the tested products (at a volume of 0.3 cm^3), according to the scheme given in the table.

Before the experiment, all the laboratory animals stayed in quarantine for 14 days. The experimental mice were subjected to daily clinical examination and control weighing. On day 28 postadministration, the animals were decapitated and pathological material (organs and blood serum) was taken for further tests.

All animal experiments were conducted in strict compliance with Interstate Standards GOST 33215-2014, GOST 33216-2014 as adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

The mass index of organs is calculated as the ratio of the organs mass to animal body mass (organ mass index = m_{organ}/m_{animal}).

Calculating titers of rabies virus-neutralizing antibodies. In order to observe adjuvant properties of chitosan-based preparations, we measured the level of rabies virus-neutralizing antibodies in sera from laboratory animals using diffusion precipitation test, according to the instruction for "Diffusion precipitation test kit for rabies diagnosis" produced by the All-Russian Research and Technological Institute of Biological Industry (Russia).

Processing statistics. The obtained statistics were processed using the standard Microsoft Excel 2007 program

adopted in biology and medicine. The results were considered reliable at the probability level of $p < 0.05$.

RESULTS AND DISCUSSION

Preliminary tests in PT-80 cell line have shown that edible chitosan (acid-soluble) at a concentration of 1:4 has a cytotoxic effect [6], therefore, during the experiment 1:64 and $1:10^8$ concentrations were used in the lab animals.

During the observation, no deviations in mice behavior were reported, and there were no specific death cases. Further autopsy revealed no pathological lesions at the injection sites (inflammation, granulation, etc.) or in the internal organs [6].

The liver is an organ that increases in size due to acute toxicity and decreases in size due to chronic toxicity. Therefore, the organ mass index was calculated.

Figure 1 shows that the mean liver mass index in all mice groups ranged from 0.045 to 0.050. This suggests that the tested preparations do not have any acute toxicity effects.

The spleen is the largest lymphoid organ. Therefore, to assess the immunity response to the administration of the tested preparations, the organ mass index was calculated for experimental mice.

The mean spleen mass index calculated (Fig. 2) for groups 1–8 and 10–16, either corresponded to the data obtained for groups 19, 20 and 37, or exceeded them ($p \leq 0.05$). This may suggest that the mice's immune system is stimulated by the chitosan-containing preparations. However, in group No. 9, the mean spleen mass index was lower than in the control groups. It can be assumed that intramuscularly administered edible chitosan (water-soluble) at a concentration of 1:64 does not have any pronounced immunostimulating effect.

To confirm chitosan adjuvant properties, the next step was to measure the level of rabies virus-neutralizing antibodies in the sera from laboratory mice using diffusion precipitation test. The experiment results are given in Figure 3.

The data obtained show that the titres of rabies virus-neutralizing antibodies post-vaccination without the tested preparations (groups No. 19 and 20) were 1:32; whereas those groups that were subcutaneously vaccinated together with water-soluble chitosan (succinate) in all the tested concentrations (groups No. 2, 4 and 6) showed the antibody level of 1:64. Regardless of the administration route, edible chitosan (acid-soluble) at all the tested concentrations (groups No. 13–16) and edible chitosan (water-soluble) at a concentration of $1:10^8$ (groups No. 11 and 12) stimulate antibody production.

Water-soluble chitosan (succinate) was administered intramuscularly at all the tested concentrations (groups No. 1, 3 and 5), edible chitosan (water-soluble) was administered subcutaneously (concentration 1:4, group No. 8) and intramuscularly (concentration 1:64, group No. 9) reduced the level of rabies virus-neutralizing antibodies down to 1:8 – 1:16. In this regard, it can be assumed that the tested preparations (at the given concentrations and administered using the mentioned routes) suppress the immune response, since natural chitosan salts are practically insoluble at pH above 6, which may be problematic for the delivery of vaccine antigens that are soluble and stable at neutral pH or higher [2].

Table
Routes of administration of the tested products to the mice groups

Preparation	Dilution	Group number	Administration route
Experimental groups			
Preparations with vaccine RABIKOV			
Preparation No. 1	1:4	1	intramuscularly
		2	subcutaneously
	1:64	3	intramuscularly
		4	subcutaneously
	$1:10^8$	5	intramuscularly
		6	subcutaneously
Preparation No. 2	1:4	7	intramuscularly
		8	subcutaneously
	1:64	9	intramuscularly
		10	subcutaneously
	$1:10^8$	11	intramuscularly
		12	subcutaneously
Preparation No. 3	1:64	13	intramuscularly
		14	subcutaneously
	$1:10^8$	15	intramuscularly
		16	subcutaneously
Control groups			
Saline solution			
Saline solution (NaCl 0.9%)	–	17	intramuscularly
	–	18	subcutaneously
Vaccine control			
Vaccine RABIKOV	–	19	subcutaneously
Vaccine RABIES	–	20	subcutaneously
Tested products without a vaccine			
Preparation No. 1	1:4	21	intramuscularly
		22	subcutaneously
	1:64	23	intramuscularly
		24	subcutaneously
	$1:10^8$	25	intramuscularly
		26	subcutaneously
Preparation No. 2	1:4	27	intramuscularly
		28	subcutaneously
	1:64	29	intramuscularly
		30	subcutaneously
	$1:10^8$	31	intramuscularly
		32	subcutaneously
Preparation No. 3	1:64	33	intramuscularly
		34	subcutaneously
	$1:10^8$	35	intramuscularly
		36	subcutaneously
No products administered (intact)	–	37	–

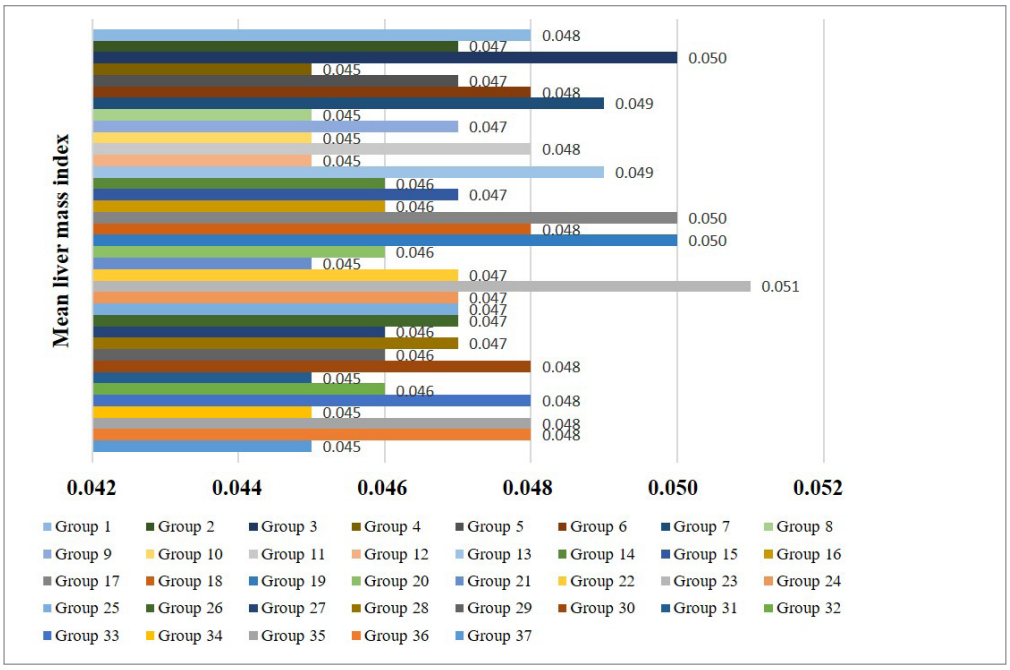

Fig. 1. Liver/body weight ratio in the laboratory mice after administration of the tested forms and concentrations of the chitosan-based products

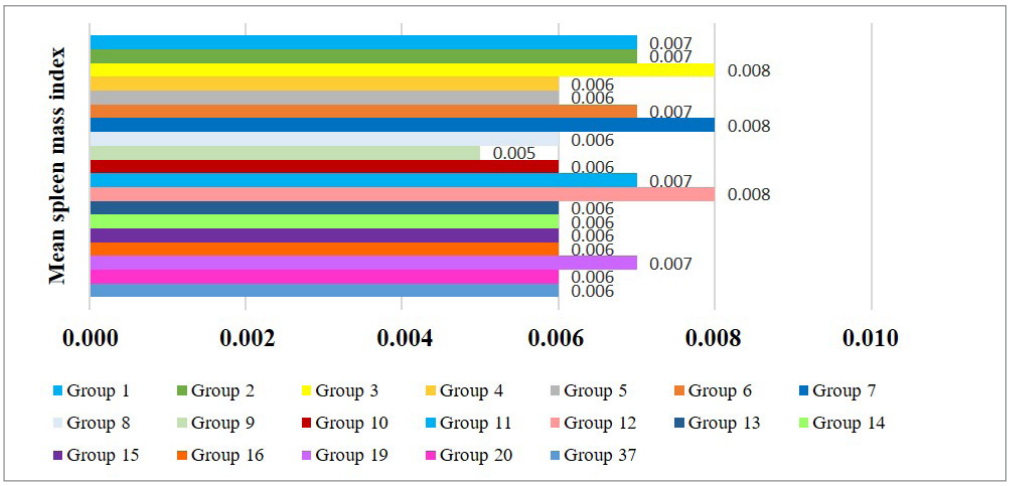

Fig. 2. Spleen/body weight ratio in the laboratory mice after administration of the tested forms and concentrations of the chitosan-based products

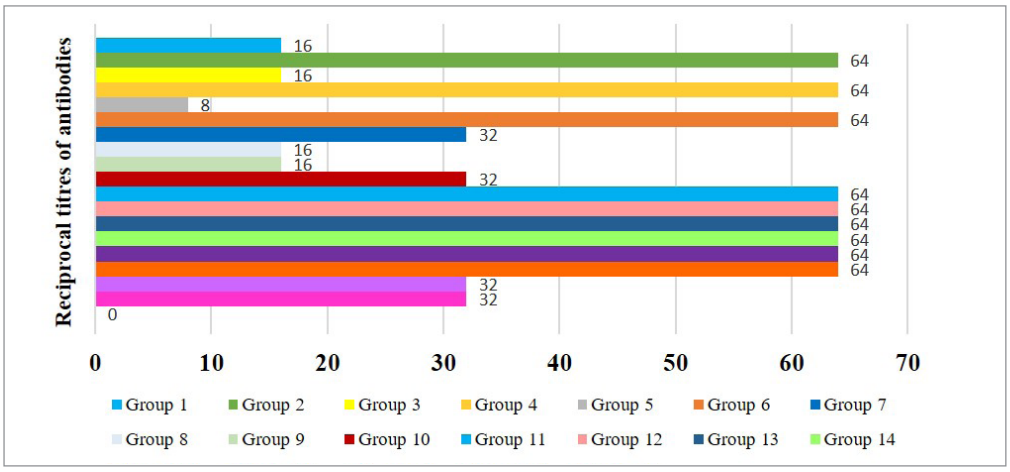

Fig. 3. Post-vaccination antibody titres against rabies virus antigen as demonstrated by the diffusion precipitation test

CONCLUSION

Thus, it has been shown that the tested chitosan-containing preparations do not have a negative impact on the laboratory animals and have immunogenic properties.

The following preparations can be recommended as affordable adjuvants: water-soluble chitosan (succinate) for subcutaneous administration; edible chitosan (acid-soluble) at a concentration of 1:64 and above, as well as edible chitosan (water-soluble) at a concentration of $1:10^8$ (administered subcutaneously and intramuscularly). Alongside it, our test results as well as results provided by other researchers [2] show that water-soluble chitosan (succinate) administered intramuscularly and edible chitosan (water-soluble) administered subcutaneously at a concentrations of 1:4 and administered intramuscularly at a concentration of 1:64 reduce the vaccine efficacy.

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