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COMPARISON OF ADJUVANT PROPERTIES OF CHITOSAN DURING ORAL AND SUBCUTANEOUS IMMUNIZATION OF MICE WITH BSA

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Vaccination is the best method to prevent the spread of infectious diseases, its disadvantages are side effects. Potentially safe DNA, RNA or protein molecules possess antigenic properties, but are low-immunogenic and therefore require conjugation with an adjuvant. The aim of the research was to evaluate Chitosan (CS) potency as an adjuvant and compare its effectiveness depending on the route of drug administration. The experiments were carried out on 3 groups of BALB/c mice. Mice of the first group were injected subcutaneously with 20 µl of a mixture of CS (3.3 mg/kg) and BSA (1.7 mg/kg). The mixture of CS and BSA at the same doses and volume was administered orally to mice of the second experimental group. The third group – control – unvaccinated mice. Anti-BSA antibody levels were measured by ELISA. Aspartate aminotransferase, alanine aminotransferase activity and cholesterol, creatinine and urea levels were determined in the serum. It was found that both subcutaneous and mucosal immunizations provided a 2-fold increase in anti-BSA antibody titers against the background of maintaining all biochemical blood parameters at the level of the physiological norm. However, AST activity in the serum of oral-immunized mice was elevated as compared to subcutaneous-immunized mice. Serum cholesterol level in the group of subcutaneously immunized mice and creatinine and urea levels in both experimental groups were reduced compared to the control. It is concluded that oral immunization with CS is the optimal route for antigen-specific IgG antibody response induction.

K e y w o r d s: vaccine development, adjuvant, antibodies, ELISA, blood biochemical parameters.

No vaccine is 100 percent safe. They have side effects: allergy, muscle pain, headache, swelling, shivering, and even mild fever. In rare cases, the vaccine might infect the patient with the virus and cause greater illness. The MMR (Measles-Mumps-Rubella) vaccine increases the risk of febrile seizures two weeks after immunization, when fever commonly occurs [1]. Serious hypersensitivity reactions have been reported following hepatitis B immunization [1]. Fatal disseminated BCG (Bacille Calmette-Guérin) infection can occur after immunization [1]. Fukuda et al. (1994) determined a biological mechanism for deafness following vaccination with attenuated measles vaccine [2, 3]. Diphtheria-tetanus-pertussis (DTP) vaccinations also significantly increase the risk of febrile seizures [4]. Safe individual antigen molecules (specific DNA/RNA sequences or proteins) have low immunogenicity and therefore require conjugation with a compound (adjuvant) to achieve stronger immunogenic properties. All available adjuvants (both officially approved and experimental) can be subdivided into several groups: 1) mineral salts such as aluminum salts (alum); 2) emulsion adjuvants such as CFA, IFA, MF59, ASO3; 3) microparticles such as virus-like particles (VLP) which are formed by structural viral proteins that mimic the intact virus size, shape, and molecular organization with self-assembly properties [5] and virosomes, which is a type of VLP platform composed of reconstituted viral envelopes with membrane lipids and viral glycoproteins that work as a carrier system for antigens or as adjuvants [6]; 4) immune potentiators such asMPL, flagellin, imiquimod, resiquimod, CpG oligodeoxynucleotides (CpG ODN), and muramyl dipeptide (MDP) [7]; 5) polyelectrolytes-ionic macromolecules [8-10]; 6) polyphosphazenes which are designed around the biodegradable phosphorus-ni-

trogen backbone and organic side groups containing anionic moieties and administered with a variety of bacterial and viral antigens in a more than a dozen of animal models [9, 11-15]; 7) polystyrene nanoparticles and nanocrystals also have adjuvant properties [16, 17]; 8) mucosal adjuvants such as chitosan (CS) which is biodegradable, biocompatible, and nontoxic [18]. Chitosan is known to induce adaptive immune responses. Low molecular weight chitosan caused heightened immunoglobulin G production in mice [19]. Vaccine efficacy depends on both adjuvant type and delivery method. Therefore, it is very important to investigate the optimal administration route for immunization. Intranasal dry powder anthrax vaccine is a positive example of effective needle free vaccine [20]. Intranasal vaccination against influenza virus also provides better immune protection than its subcutaneous injection in mice [21]. Wong-Chew and colleagues found out that aerosolized and subcutaneous measles vaccines are equally effective in children [22]. The aerosol measles vaccine had higher immunogenicity than subcutaneous injection in school-age children [23]. An oral administration of bite salt-incorporated lipid vesicles containing influenza A antigen stimulated higher antibody titers than intramuscular injection in mice [24]. CS with low molecular weight and high molecular weight were investigated for their ability to act as adjuvants during protein vaccination in a mouse model [19]. The impacts of low molecular weight and high molecular weight CS on anti-OVA IgG titers productions were examined after intramuscular immunization. It was declared that low molecular weight CS, but not high molecular weight CS, as an adjuvant is able to significantly increase IgG level after immunization [19]. Taking into account these data we had chosen low molecular CS for our experiments. An important demand for adjuvants is that they do not cause side reactions which can be detected via biochemical analysis of the blood. Chitosan decreased the serum levels of AST, ALT, and ALP which significantly improved hepatic fibrosis [25]. The purpose of this study was to evaluate and compare adjuvant properties of low molecular weight CS in oral and subcutaneous injection routes and measure blood biochemical profiles.

Materials and Methods

Chitosan (448869) – low molecular weight (50,000-190,000 Da) was purchased from Sigma-Aldrich, Germany. The amino groups of chitosan (Fig. 1) are crucial for protein binding.

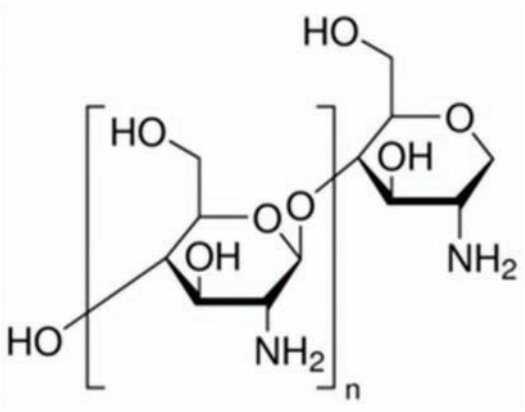

Fig. 1. The chemical structure of chitosan (Sigma-Aldrich, Germany)

Ethics statement. The protocol for animal experiments was approved by the Ethical Committee of the Institute of Animal Biology of NAAS of Ukraine and the experiments were carried out in accordance with the European Convention for the Protection of Vertebrate Animals (Strasbourg, 1986).

Mice. White laboratory BALB/c mice (*Mus musculus*) were provided by the State Scientific-Research Control Institute of Veterinary Medicinal Products and Feed Additives. Mice were maintained in a specific pathogen-free animal facility with water and commercial food (Lux, Poland) provided *ad libitum*.

Immunization. Mice immunization was done using white laboratory BALB/c mice, both male and female. Animals of 2 months of age were divided into three groups of five mice each. Mice of the first experimental group were injected subcutaneously (the site for injection is over the shoulders) with mixture (20 µl) of the chitosan (3.3 mg/kg) and BSA (1.7 mg/kg) (Merck, Germany); the same doses and volume of chitosan and BSA were administered orally to mice of the second experimental group. Control mice were unvaccinated. Immunization was performed on days 1, 14 and 28. The solutions were sterilized by autoclaving. Two weeks after the last injection, animals were anesthetized in an induction chamber for 5 sec using chloroform (Sphere Sim, Ukraine), decapitated by cervical dislocation and their blood was taken for analysis. Blood was collected in 1.5 ml Eppendorf tube containing 7.5 ul Heparin 1000 u/ml.

Antibody purification. Immunoglobulins were isolated from the blood serum of mice by precipitating three times with a saturated solution of ammo-

nium sulfate (Sphere Sim, Ukraine). Immunoglobulins were then dissolved in phosphate-buffered saline (PBS).

ELISA method: 100 µl of 1% BSA solution was adsorbed onto a 96-well plate (PAA, Austria), incubated for 24 h at 4°C; the wells were then washed three times with buffer A (0.2% BSA in PBS) isolated immunoglobulins in solution were added to the wells and incubated for 2 h at 37°C; then washed three times with buffer A, conjugated anti-mouse antibodies (Sigma, Germany) were added in a 1: 5000 dilution, incubated for 1 h at 37°C; the wells were then washed three times with PBS-Tween-20, and the substrate for alkaline phosphatase, p-nitrophenylphosphate in diethanolamine (Filisit-Diagnostics, Ukraine) was added; after incubating at room temperature for 3 min, absorbance at 405 nm was measured on an ELISA plate reader (STAT FAX Awareness Technology Inc., USA).

Biochemical analyses in blood serum. The activity of aspartate aminotransferase (AST; EC 2.6.1.1), alanine aminotransferase (ALT; EC 2.6.1.2), and contents of cholesterol, creatinine, urea, total protein and albumin were determined in the serum of animals. Blood chemistry tests were performed on the Humalyzer-2000, Germany.

Statistical analysis. Statistical calculations of results ($M \pm m$) were performed using Microsoft Excel 2007. The probability of differences was determined by the Student's *t*-test with $P < 0.05$ accepted as statistically significant.

Results and Discussion

Chitosan (CS) is a promising immune modulator (adjuvant) for modern vaccines. We compared administration routes (oral and subcutaneous) on mice using BSA as a model antigen. Mice of the first experimental group were injected subcutaneously with CS+BSA. Mice of the second experimental group were immunized orally with CS+BSA. It was found that mucosal administration was more effective in individual cases than subcutaneous. Statistical analysis of the results showed similar levels of anti-BSA antibodies for both administration routs (Fig. 2). Subcutaneous and oral immunizations provided a 2-fold increase in anti-BSA antibody titers (Fig. 2). The immunization of mice only with antigen (ovalbumin or BSA) without adjuvant did not cause specific antibody production [26].

In view of the fact that the oral immunization with low molecular weight chitosan is a painless procedure, it deserves more attention for the development of safe vaccines.

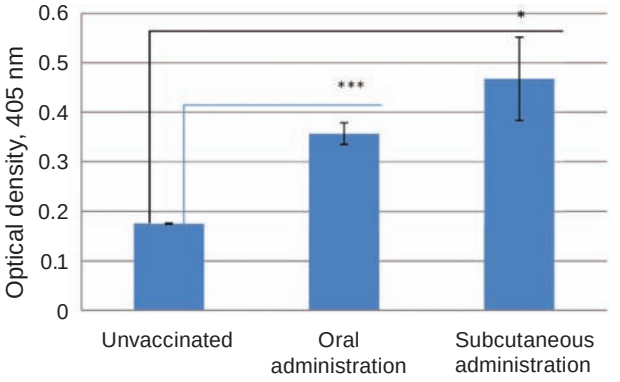

*Fig. 2. ELISA analysis of anti-BSA antibodies level in the serum of mice. *P < 0.05, ***P < 0.001 statistically significant*

There were some differences in food intake in the oral-immunized animals compared to the control group and subcutaneous-immunized mice. Oral-immunized mice had greater appetites than others. Their total weight was 15.1% higher than subcutaneous-immunized mice at the end of the experiment. Organ-to-body weight ratio have long been accepted as a sensitive indicator of induced changes to organs. The organ-to-body weight ratios of the liver, kidney, and spleen of animals immunized via oral and subcutaneous routes did not show any significant differences from the control group (Fig. 3), and between the groups.

The results of this study showed that the immunizations were safe because each did not cause lower mean mouse weight. Slight increase in body weight

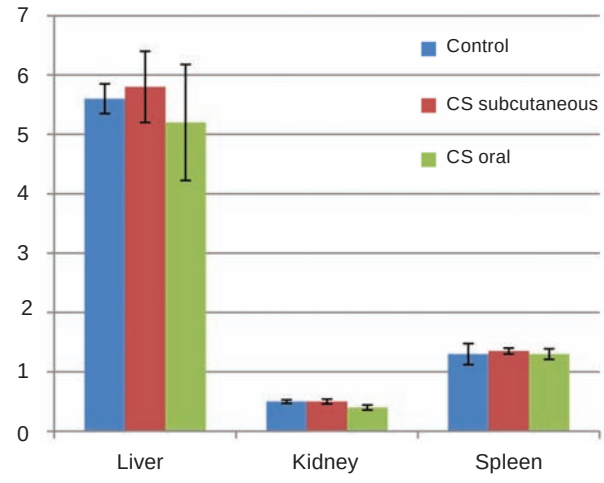

Fig. 3. Organ-to-body weight ratios (liver, kidney and spleen). CS – low molecular weight chitosan

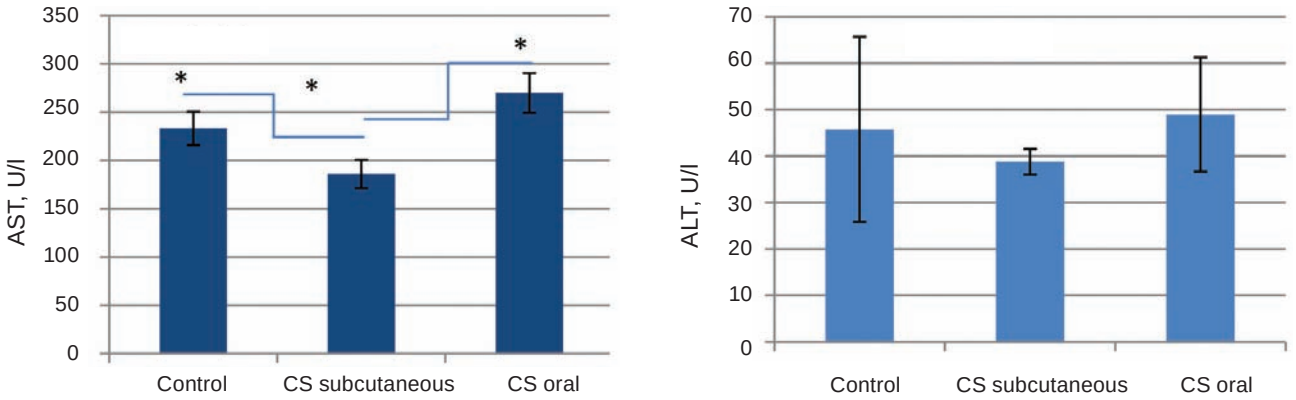

*Fig. 4. Aspartate aminotransferase (AST) activity and alanine transaminase activity (ALT) in the serum of mice, U/l. SC – low molecular weight chitosan, * P < 0.05, **P < 0.01 statistically significant*

is a good sign according to Nnamdi et al. [27, 28]. Perkins et al. showed that the pertussis-containing vaccines that cause weight loss could cause adverse reactions in children [28].

We analyzed the activities of the enzymes that are markers for liver functions. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in serum of vaccinated and control mice. AST activity was elevated in oral-immunized mice compared to subcutaneous-immunized mice (Fig. 3). Subcutaneous-immunized mice also have lower AST activity than control mice. Despite the differences, AST was in physiological range for all mice [29]. Zheng et al. reported that low molecular weight CS has dual activities, both immunostimulatory activity and anti-inflammatory [30, 31]. We suggest that the anti-inflammatory functions of CS could explain AST decreasing.

ALT levels in the control group were not significantly different from immunized mice (Fig. 4).

Cholesterol content in the blood of mice differed little between all groups (Fig. 5). Cholesterol levels were significantly ($P < 0.05$) decreased in subcutaneous-immunized mice compared to the control. However, the observed cholesterol levels were within the normal mice physiological range [29].

Creatinine was significantly lower in both experimental groups, but it was within the normal mice physiological range [29] (Fig. 6).

Blood urea contents were significantly lower in immunized mice (Fig. 6), but they were within the normal mice physiological range [32].

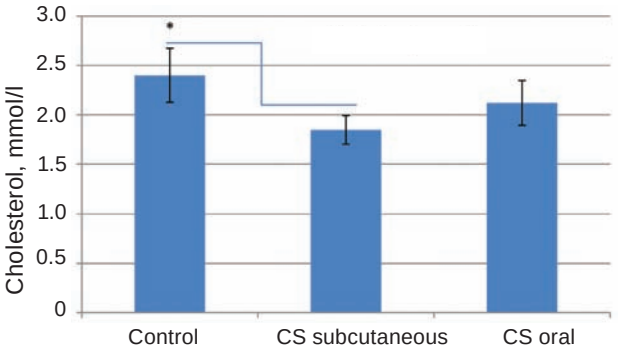

*Fig. 5. Cholesterol content in the serum of mice, mmol/l. SC – low molecular weight chitosan, *P < 0.05 statistically significant*

Conclusions. Low molecular weight Chitosan as adjuvant caused 2-fold increased specific antibody levels in mice after oral and subcutaneous immunizations. The advantage of oral vaccination is that it is needle-free painless administration. Organ-to-body weight ratio and biochemical analyses demonstrate the safety of chitosan as an excellent adjuvant for the development of safe and effective vaccines.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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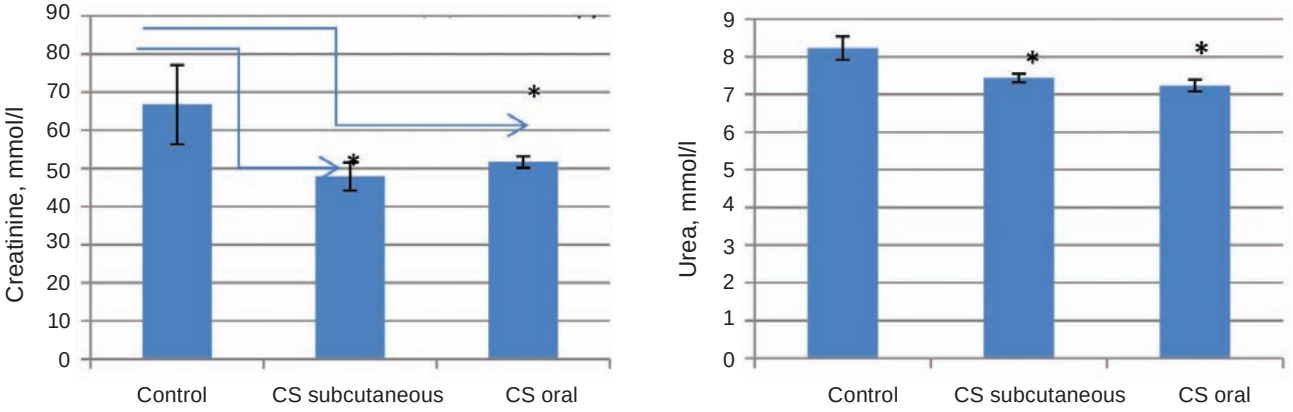

*Fig. 6. Creatinine and urea content in the serum of mice, mmol/l. SC – low molecular weight chitosan, *P < 0.05 statistically significant*

ПОРІВНЯННЯ АД'ЮВАНТНИХ ВЛАСТИВОСТЕЙ ХІТОЗАНУ ЗА ПЕРОРАЛЬНОЇ ТА ПІДШКІРНОЇ ІМУНІЗАЦІЇ МИШЕЙ БСА

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Вакцинація – найкращий спосіб для запобігання поширення інфекційних захворювань. Недоліками вакцинації є її побічні ефекти. Молекули ДНК, РНК або протеїни, які мають антигенні властивості, є потенційно безпечними. Однак ці молекули мають низьку імуногенність, і тому потребують кон'югації з ад'ювантом. Метою дослідження було оцінити потенціал хітозану як ад'юванта та порівняти його ефективність за підшкірного та орального способу введення. Експерименти проводили на 3 групах мишей лінії BALB/c. Мишам першої групи вводили 20 мкл суміші хітозану (3,3 мг/кг) та БСА (1,7 мг/кг) у забуференому фізіологічному розчині підшкірно. Мишам другої дослідної групи суміш хітозану та БСА за тих же доз і об'ємів вводили орально. Третя група – контрольна – не вакциновані миші. Імунізацію проводили на 1, 14 та 28 дні експерименту. Рівень антитіл до БСА визначали методом ELISA. У сироватці крові визначали активність аспартатамінотрансферази та аланінамінотрансферази, вміст холестерину, креатиніну та сечовини.

Встановлено, що як підшкірна, так і оральна імунізація забезпечувала 2-кратне підвищення титрів анти-BSA антитіл на фоні утримання усіх біохімічних показників крові на рівні фізіологічної норми. Однак у сироватці мишей, імунізованих орально, активність аспартатамінотрансферази була підвищеною порівняно з мишами, імунізованими підшкірно. Зниженими виявились рівень холестерину у сироватці мишей, імунізованих підшкірно та креатиніну і сечовини в обох експериментальних групах порівняно з контролем. Зроблено висновок, що оральна імунізація з хітозаном є оптимальним шляхом для індукції антиген-специфічних антитіл.

Ключові слова: розробка вакцини, ад'ювант, антитіла, імуноензимний аналіз, біохімічні показники крові.

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