Adjuvant effect of Shilajit and plant extracts on the immune responses to bovine serum albumin in hens

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Abstract

The aim of current study was to test an immunostimulator activity of three different components such as Shilajit, Hedysarum neglectum and Ginkgo biloba that correspond to A, B and C groups respectively. Three groups of hens were immunized orally with a bovine serum albumin (BSA) three times once a week. Serum and saliva were collected every 7th day during 9 weeks. Eggs were taken every day throughout the experiment. BSA-specific immunoglobulin A (IgA) antibodies in saliva, IgM antibodies in serum and IgY antibodies in egg yolk were examined by ELISA. A total IgY (mg/ml) was measured using a photometric technique. We observed an immense increase of IgA titres in two groups that were immunized with Shilajit and H. neglectum. Immunoglobulin Y titres were higher in the group A. However the total IgY is mounted four times more in the group B. Immunoglobulin M titres were at the similar level throughout the observation period. Interestingly, G. biloba showed the poorest Ig titres. Summarizing the named herbomineral and two medicinal plants can be advised to boost the immune response in birds.

Key words: *Shilajit, Hedysarum neglectum, Ginkgo biloba, hens, oral administration, mucosal immunity.*

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Introduction

Organic compounds and substances are represented as an important source for new drug research. There is an ever-growing interest to a different species of plants to identify their potential therapeutic applications such as immunomodulatory activity, biological and medicinal properties [1-10].

Shilajit (common names are mumie or vegetable asphalt) is a herbomineral substance that consists of 60-80% of organic, 20-40% of mineral material and 5% of trace elements. Actually it is a semi-hard brownish black resin formed through long-term humification of *Euphorbia* sp. and *Trifolium* sp. plants. Shilajit is widely used as a part of the ayuverdic medicine for many years [4]. Shilajit is one of the perspective substances that can be used in traditional medicine.

It was reported recently that root of *Hedysarum neglectum* contain several interesting compounds as mangiferin, tannins and some polysteroids [11]. Although the effect of all these compounds is not well understood it was shown an ability of mangiferin to stimulate immune system [12].

Ginkgo biloba leafs contain numerous active compounds mainly flavonoids and triterpenes [13]. An extract from leafs is widely used in various compositions of commercial additives [14]. It is recommended to use the *G. biloba* extract for maintaining different functions in an organism. Interestingly the evaluation of capacity to boost immune system has been started recently. In 2004 it was reported that extract of *G. biloba* was effective in stimulation of humoral immune response in rats [15]. Later, a successful study was conducted to evaluate the same extract on immunity activation in human [16].

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Few trials focusing on immunomodulation ability of Shilajit, *H. neglectum* and *G. biloba* were performed. On the base of previous results it is supposed that they can boost the immune response. Elevation of immunoglobulin titres is a sign of immune system activation. Secretory immunoglobulin A (IgA) play a central role in mucosal immunity that is established *via* the mucosa-associated lymphoid tissues mainly in respiratory and gastrointestinal tracts [17]. The gut-associated lymphoid tissues (GALT), particularly the Peyer's patches contain a large number of IgA precursor B cells. The stimulation of them by orally administered antigens lead to dissemination of B and T cells to mucosal effector tissues and various secretory glands that consequently induce an antigen-specific IgA response [18].

Serum IgM is the first antibody that appears in response to an antigen. It can activate complement *via* classical pathway [19]. An increase of IgM is a sign of immune system activation. Immunoglobulin Y (IgY) is a hen's analog of mammalian IgG that is found in egg yolk [20]. It transfers from mother to offspring with a purpose to protect an offspring during the first days of their life [21]. Initially IgY has antigen-specificity to those antigens that were exposed to mother organism [22]. There is an increasing interest in the use of chicken egg yolk antibodies IgY for scientific and diagnostic purposes [23]. Application of IgY technology provides an opportunity to get antigen-specific antibodies in a large amount and during long period [20].

In the present study we examined the immunomodulation capability of herbomineral Shilajit and two medicinal plants as *H. neglectum* and *G. biloba* at a concentration of 133 mg/kg, 16 mg/kg and 105 mg/kg respectively in laying hens model. Bovine serum albumin (BSA) was employed as antigenic substance for bird's organism [24, 25]. Additionally the efficacy of large-scale specific IgY production was assessed.

Material and methods

A commercial preparation of BSA (Sigma, USA) was used as a model antigen.

Shilajit (Evalar, Russia), dry root of *Hedysarum neglectum* (Evalar, Russia) and *Ginkgo biloba* leaves (Ginkgo Biloba Pharma Nord ApS, Denmark) were used as prospective adjuvants.

Hens

Nine 25-week-old outbreed ISA hens (1.5 ±0.05 kg of weight) were obtained from the breeding unit of the laboratory of animal resources at the State Research Institute Center for Innovative Medicine (Vilnius, Lithuania). Hens were kept singly in 1 m × 1 m floor pens equipped with nest boxes, in a standard animal room with a 17/7 h light/dark cycle (light long 25 lux). As bedding, chips of deciduous trees were used processed by sterilisation at 120°C and pressure 1.5 kg/cm^2 during for 20 min. The bedding was changed twice a week. A temperature in the room was 16 ±2°C, with a relative humidity within the range of 60-70%. Chicken feed was based on granulated forage ("Kauno grūdai" AB Kaunas, Lithuania) which consisted of energetic (11.7 MJ/kg), crude protein (17%), crude oil (3%), and crude fibre (71%). The feed was balanced for vitamins and micronutrients, and amino acids. Water was provided *ad libitum.* We performed the experiment having the permission from the Ethics Committee on the Use of Laboratory Animals, at the State Food and Veterinary Service of Republic of Lithuania (Permission No. 0209).

Immunization groups

Nine animals were involved in the study. They were divided into three treatment groups A, B and C containing three chickens in one group. The amount of animals in each experimental group was selected according the protocols presented by Prof. J. Hau [26].

An immunization mixture contains 200 mg of BSA that was diluted in emulsion of 0.5 ml of phosphate-buffered saline (PBS), pH 7.2 and 0.5 ml of olive oil (Extra Virgin, Carapelli Firenze, Italy). The immunostimulatory components were added additionally.

Group A. 200 mg of Shilajit was added to the 1 ml of immunization mixture that corresponds to a dose 133 mg/kg.

Group B. The BSA containing emulsion (1 ml) was mixed with 1 ml 20% hydro-alcoholic extract of *H. neglectum* that corresponds to a dose 16 mg/kg.

Group C. 157 mg of dry extract from *G. biloba* leaves was diluted in 1 ml BSA containing immunization mixture that corresponds to a dose 105 mg/kg.

Procedures

Birds in groups A, B and C were immunized *via* oral gavage on days 1, 7 and 14.

For oral administration was used plastic syringe (Luer) where hub was supplemented with a feeding needle (length 75 mm, width 15 mm) with silicon tip at the end. The procedure was carried out by administering the immunization mix by esophageal intubation. Generally the immunization *via* oral gavage is welcomed strategy for routine production of antibodies [27]. Moreover this procedure is less stressful for an animal that is performed without anesthesia [28].

Saliva was collected in absorbent filter papers (Whatman No. 1, Sigma). Pre-weighed two wicks were placed under the tongue of the hen for 20 s. The wicks were weighed after that again. An amount of saliva represents in difference between two weightings. Saliva was extracted by adding 400 µl of PBS containing 0.1% Tween 20, pH 7.2, to the Eppendorf tube with the paper wicks and incubating the mixture with slow shaking for 2 h at 20°C.

Serum was collected from the clotted blood obtained from the wing vein and stored at –20°C until use. It is known that serum is stable until 90 days under mentioned conditions [29].

Eggs were collected every time when they were layed and then pooled in one sample for individual chicken at each single week.

BSA-specific antibodies IgA in saliva, IgM in serum and IgY in egg yolk were measured in all three groups and were examined by ELISA as described earlier [30]. The antibody titres were expressed, as the reciprocal of the highest dilution of saliva, serum and egg yolk at the optical density of $\lambda = 492$ nm (Titertek Multiscan Plus MK II, Labsystems Finland), which was 2-fold higher than that of the negative samples. Antibody titres were converted into a base-2 logarithmic scale. The purification of IgY from egg yolk was performed as described earlier [31]. The protein content was measured photometrically at $\lambda = 280$ nm (BioPhotometer, Eppendorf, Germany) and recalculated according to the Lambert-Beer law with an extinction coefficient for IgY of as 1.34. Total amount of IgY was expressed in mg per ml.

Statistical analysis

Statistical evaluation of the results was done by one-way analysis of variance ANOVA using PRISM Software (GraphPad Software, San Diego, CA, USA). The mean of the IgA, IgY and IgM antibody titres as well as the total IgY content were compared using unpaired Student's *t*-test with Welch's correction. All values were expressed as mean ± standard deviation and were considered to be statistically significant at $P < 0.05$.

Results

Immunoglobulin A titres increased after the third immunization in all three groups (Fig. 1). However the increase of group C was less than in groups A and B. The IgA titres of group C were at a similar level during the whole experiment while the IgA titres of groups A and B were slightly raising until the 6 week. Immunoglobulin M titres increased in all tested groups and remained at the same level during the whole observation period (Fig. 2).

Immunoglobulin Y titres elevated drastically after the last immunization (Fig. 3). It was recorded a variation of IgY titres from 12 to 16 in groups A and B. However the response in group A was stronger where IgY titres peaked on the fourth and fifth weeks. Immunoglobulin Y titres as IgA titres were the lowest in group C. A time-dependent fluctuation in total amount of IgY is shown on Fig. 4. Unexpectedly high elevation was detected in group B. The total IgY amount reached 75 mg/ml at the first week and fluctuated 65 ±10 mg/ml during the whole experiment. In the other hand the total amount of IgY was similar in groups A and C and varied 20 ±10 mg/ml.

Summarizing we observed stimulation of immune response in chickens after three immunizations with BSA combining with three different components. It was discovered that Shilajit and *H. neglectum* are more efficient immunostimulators than *G. biloba.* Furthermore *H. neglectum* showed to be efficient in large-scale production of antigen-specific immunoglobulin.

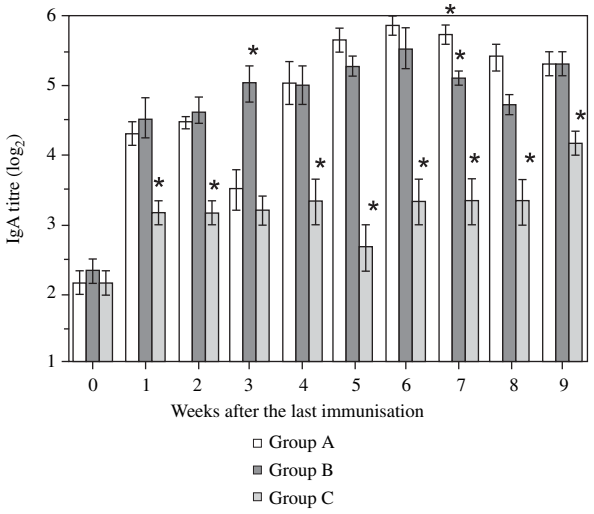

Fig. 1. Saliva IgA antibody response in hens after oral immunizations with bovine serum albumin (BSA) in combination with different adjuvants. The asterisks indicate groups the values of which differ significantly from the other ones in the same week; *statistically significant $P < 0.05$

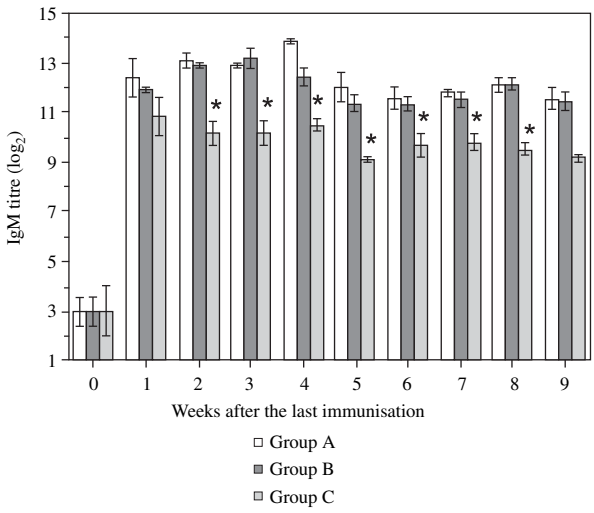

Fig. 2. Serum immunoglobulin M (IgM) antibody response in hens after oral immunisations with bovine serum albumin (BSA) in combination with different adjuvants. The asterisks indicate groups the values of which differ significantly from the other ones in the same week; *statistically significant $P < 0.05$

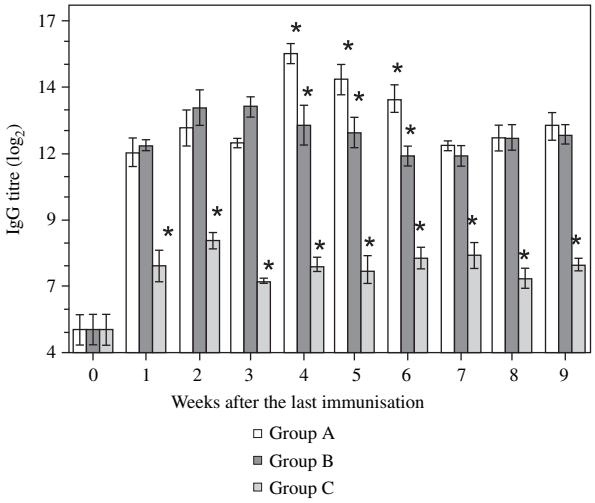

Fig. 3. Yolk immunoglobulin G (IgG) antibody response in hens after oral immunisations with bovine serum albumin (BSA) in combination with different adjuvants. The asterisks indicate groups the values of which differ significantly from the other ones in the same week; *statistically significant $P < 0.05$

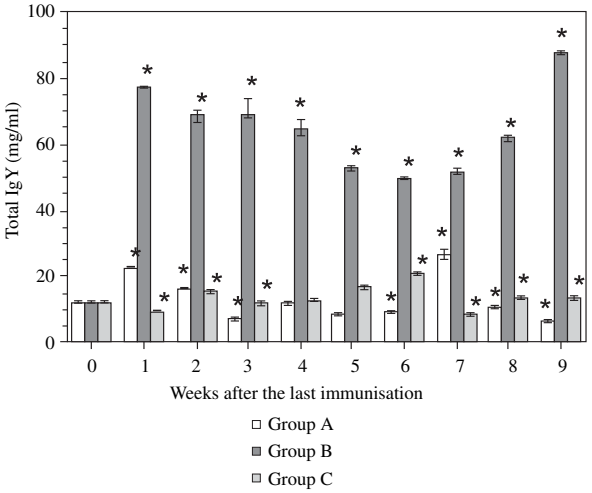

Fig. 4. Time-dependent changes in the total immunoglobulin Y (IgY) content. The asterisks indicate groups the values of which differ significantly from the other ones in the same week; *statistically significant $P < 0.05$

Discussion

An induction of a systemic humoral immune response is complicated. A combination of antigen, boosting element and immunization protocol has to be precisely selected. Our model of study is tightly composed and corresponds to the other studies where BSA was used as the antigen for provoking immune response in chickens [24, 25]. Firstly, an antigenic capacity of BSA is enough to cause gentle immune response [32, 33], but usually it is combined with immunostimulator as boosting element. Secondly, the immunization mixture can be enriched additionally by emulsifying it with an oil to provoke a prolonged enhancing effect [34]. It occurs due to the position of soluble antigen in the internal aqueous phase that provides slow release into biological fluids. Furthermore, an antigen administration *via* oral immunization causes a reactivation of humoral as well as cellular components of immune system. We assessed the immunostimulatory capacity of Shilajit and two extracts from *H. neglectum* and *G. biloba* under the described conditions.

Our findings present that all three components can be advised for the immunostimulating application as adjuvants in chicken model. It is shown that the developed immune response can persist for longer period, even until 3 months, after the immunization. The herbomineral Shilajit and the extract from *H. neglectum* showed similar efficacy to boost immune response while surprisingly the extract from *G. biloba* was less effective. These data supplement previous reports about plant potency for the mammalian organism [35-40].

Additionally we evaluated the outcome of the antigen-specific IgY content that was transferred into egg yolk. It is possible to get 75 mg per ml of polyclonal antigen-specific immunoglobulin using extract of *H. neglectum* as mucosal immunostimulator in chicken model. High yield of IgY persists in egg yolk longer than one year and after it slightly decreases [41]. Although the total amount of immunoglobulin depends on a context of an antigen. This has to be taken into account for further experiments.

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