World Rabbit Sci. 2016, 24: 295-301
doi:10.4995/wrs.2016.4139

IMMUNE RESPONSES INDUCED IN RABBITS AFTER ORAL ADMINISTRATION OF BOVINE SERUM ALBUMIN IN COMBINATION WITH DIFFERENT ADJUVANTS (HERB EXTRACTS, ALUMINIUM HYDROXIDE AND PLATINUM NANOPARTICLES)

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Abstract: The aim of the current study was to evaluate the immunostimulatory activity of 10 different herbal extracts from *Vitex agnus-castus*, *Vinca major, Aloe arborescens* and the polyherbal product containing extracts from *Sambucus nigra*, *Primula versis*, *Pinus alba*, *Gentiana lutea*, *Cetraria islandica*, *Eucaliptus globulus*, *Citrus limon* and aluminium hydroxide, as well as platinum nanoparticles. Rabbits were immunized three times orally with bovine serum albumin (BSA) in combination with the components mentioned above. BSA-specific IgA antibodies in saliva and IgG antibodies in serum were examined by ELISA. It was found that the rabbits immunized with BSA in combination with either platinum nanoparticles or aluminium hydroxide had higher titres of BSA-specific IgA antibodies in their saliva at day 56 of observation. Likewise, rabbits treated with BSA and *Vinca major* or *Aloe arborescens* extracts showed higher levels of BSA-specific IgG antibodies in the serum at the end of observation. These results suggest that some plant extracts, aluminium hydroxide and platinum nanoparticles components could be used as oral adjuvants or as immunomodulators for rabbits.

Key Words: herb extract, platinum nanoparticles, aluminium hydroxide, rabbits, oral administration, immunostimulatory activity.

INTRODUCTION

Medicinal herbs play an important role in researching new substances with adjuvant-like properties and are used in veterinary vaccine formulations or as food supplements to reduce the risk of various diseases (Ragupathi *et al.,* 2008; Yang *et al.,* 2008; Patel, 2012; Moudgil *et al.,* 2015).

Particularly, adjuvants can be used to improve the immune response to antigens for several different purposes, including: 1/ increasing the immunogenicity of weak antigens; 2/ enhancing the speed and duration of the immune response; 3/ modulating antibody avidity, specificity, isotype or subclass distribution; and 4/ promoting the induction of mucosal immunity (Singh and O'Hagan, 2003). Identifying the immunostimulating properties of herbs at cellular and humoral levels has involved nearly 3 decades of research.

A variety of herb extracts were discovered that would induce the production of antibodies in various animals (Bižanov and Tamosiumas, 2005; Jonauskienė and Bižanov, 2008; Ragupathi *et al.,* 2008; Yang *et al.,* 2008; Al-Bowait *et al.,* 2010; Bižanov *et al.,* 2010; Patel and Asdaq, 2010; Popov *et al.,* 2010; Bižanov *et al.,* 2012; Xiao *et al.,* 2013).

There are several important conditions when performing analysis of extracts, including: 1/ immunization scheme; 2/ dosage; 3/ administration route, i.e. parenterally, orally, *via* drinking and feeding.

To enhance immune response and develop advanced medical strategies, different nanoparticles (platinum nanoparticles (PtNP)) (Challacombe *et al.,* 1992; Ghoneum *et al.,* 2010) or aluminium hydroxide (Cox and Coulter, 1997; Naim *et al.,* 1997) have been found to exhibit immune system stimulating activity.

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doi:10.4995/wrs.2016.4139

Extracts from *Vitex agnus-castus*, *Vinca major*, *Aloe arborescens* and the polyherbal product containing extracts from *Sambucus nigra*, *Primula versis, Pinus alba*, *Gentiana lutea*, *Cetraria islandica*, *Eucaliptus globulus*, *Citrus limon* have not been previously examined as potential immunoadjuvants after oral administration.

The aim of the present study was to assess the efficacy of rabbits treated orally with bovine serum albumin (BSA) in combination with several components: platinum nanoparticles, aluminium hydroxide, herb extract from *Vitex agnus-castus*, *Vinca major*, *Aloe arborescens* and the polyherbal product containing extracts from *Sambucus nigra*, *Primula versis*, *Pinus alba*, *Gentiana lutea*, *Cetraria islandica*, *Eucaliptus globulus*, *Citrus limon* as mucosal adjuvants. The efficiency of the systemic and mucosal immunospecific response was evaluated by measuring BSA antibodies (IgG in serum and IgA in saliva).

MATERIAL AND METHODS

Animals

Eighteen male Chinchilla rabbits each weighing 3.3-3.5 kg were used in the study. The rabbits were obtained from the laboratory animal resources breeding unit at the State Research Institute Centre for Innovative Medicine (Vilnius, Lithuania).

At the age of 340 d, when the experiment started, they were separated into 6 groups (A [*Vinca major*], B [*Vitex agnus-castus*], C [*Aloe arborescens*], D [platinum nanoparticles], E [$Al(OH)_3$] and F [mixture of 7 plants] 3 rabbits/group) and housed in cages (71.3×71.6×47.6 cm) (Techniplast, Italy) in standard animal rooms on a 17/7 h light/dark cycle (light long 25 lux). As bedding, chips from deciduous trees were used, after sterilization at 120°C at a pressure of 1.5 kg/cm^2 for 20 min. Bedding was changed twice a week. The room temperature was 16±2°C, with a relative humidity within the range of 55-60%. The rabbits were fed with granulated forage ("Kauno grūdai" AB Kaunas, Lithuania), which consisted of digestible energy (8.8 MJ/kg), crude protein (18.5%), crude fat (3.5%) and crude fibre (15%). The feed was balanced for vitamins, micronutrients and amino acids. Water was provided *ad libitum*. We performed the experiment after receiving permission from the Ethics Committee on the Use of Laboratory Animals at the State Food and Veterinary Institute (№ 0209).

Antigen

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

Preparation of herb extract

The extracts from *Vinca major* and *Vitex agnus-castus leaves* (containing 0.08-0.6% of glucoside agnoside) were obtained from the company "Aconitum" (Kaunas, Lithuania). Leaves of seventh-year-old *Aloe arborescens* were collected in 2008 in the greenroom of the Institute of Immunology (Vilnius, Lithuania). Then, 160 g of the *Aloe arborescens* leaves were extracted with 70% ethanol solution for 7 d at room temperature and the remaining aqueous residue was subsequently concentrated and dried at 30°C. The extracts from *Sambucus nigra*, *Primula versis*, *Pinus alba*, *Gentiana lutea*, *Cetraria islandica*, ether extracts from *Eucaliptus globulus* and *Citrus limon* were obtained from Innovative Pharma Baltic (Vilnius, Lithuania) as a polyherbal product. The herbal extracts and elements were applied to rabbits in dosages comparable to those in human therapy, i.e. calculated equivalent to human beings according to their daily dose. Similar standardization has been applied by other authors (Bonidet *et al.,* 2002; Rasool *et al.,* 2014).

Preparation of platinum nanoparticles

The PtNP were synthesized by the reduction of H_2PtCl_6 with sodium citrate in the presence of tannic acid according to a modified 13 nm gold nanoparticles synthesis, as described earlier (Slot and Geuze, 1985). For the preparation of PtNP, the initial concentration of platinum (according to mass) was 37.65 µg/mL.

Preparation of aluminium hydroxide

Solution of the $Al(OH)_3$ in PBS was obtained from Biofa Co. (Vilnius, Lithuania). When suspended in water, Al_2O_3 reacts with water (it is hydrated, so to speak) to form aluminium hydroxide, $Al(OH)_3$, in the form of an amorphous precipitate. Aluminium hydroxide is thus the name for the chemical entity $Al(OH)_3$, i.e. the hydrated form of aluminium oxide. The aluminium concentration was 1.0 mg/mL.

Experimental design

Rabbits were randomly divided into 6 groups, each with 3 rabbits. The immunization mixture contained 200 mg of BSA 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 (*Vinca major*). 5.0 mg of dry *Vinca major* extract was diluted in 1 mL PBS and mixed with 1 mL BSA containing immunization mixture corresponding to a 1.47 mg/kg dose.

Group B (*Vitex agnus-castus*). 10.0 mg of dry *Vitex agnus-castus* extract was diluted in 1 mL PBS and mixed with 1 mL BSA containing immunization mixture corresponding to a 2.94 mg/kg dose.

Group C (*Aloe arborescens*). 7.0 mg of dry *Aloe arborescens* extract was diluted 1 mL PBS and mixed with 1 mL BSA containing immunization mixture corresponding to a 2.05 mg/kg dose.

Group D (platinum nanoparticles). 47.6 µg of PtNP was diluted in 1 mL PBS and mixed with 1 mL BSA containing immunization mixture corresponding to a 19.1 µg/kg dose.

Group E ($Al(OH)_3$). 1.0 mg of $Al(OH)_3$ was diluted in 1 mL PBS and mixed with 1 mL BSA containing immunization mixture corresponding to a 0.29 mg/kg dose.

Group F (mixture of 7 plants). 10 mg of dry *Sambucus nigra* extract, 15 mg of dry *Primula versis* extract, 8 mg of dry *Pinus alba* extract, 6 mg of dry *Gentiana lutea* extract, 8 mg of dry *Cetraria islandica* extract, 120 mg of ether *Eucaliptus globulus* extract, 20 mg of ether *Citrus limon* extract was diluted 1 mL PBS and mixed with 1 mL BSA containing immunization mixture corresponding to doses of 2.9, 4.4, 2.3, 1.8, 2.3, 32.3 and 5.9 mg/kg, respectively.

Rabbits in groups A, B, C, D, E and F were administered immunization mixtures orally on days 1, 7 and 14. The rabbits were not anaesthetized during immunizations. Plastic (Luer) was used for oral administration, where hub was supplemented with a feeding needle (length 75 mm, width 15 mm) containing a silicon tip at the end as described previously (Bižanov *et al.,* 2012).

Sampling

Samples of serum and saliva were collected at 0 d and at 14, 28, 42, and 56 d after the last administration. Blood samples were taken from the marginal ear vein. The blood was allowed to clot for about 1 h at room temperature (RT). The clot was then loosened with a glass rod to promote separation and left overnight at 4°C. The clot-free liquid was transferred into a tube and centrifuged for 20 min at 1500 *g* at 4°C, as described previously (Bizhanov and Vyshniauskis, 2000).

Saliva secretion was collected using absorbent filter papers (Whatman No.1, Sigma). Two pre-weighed wicks were placed under the tongue of the rabbit for approximately 20 s. The wicks were weighed to measure the amount of saliva. The 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 by slowly shaking at 20°C for 2 h. The extract obtained was subsequently used for analysis.

ELISA

BSA-specific IgA antibodies in saliva and BSA-specific IgG antibodies in serum from all 5 groups were examined by ELISA. Microtitre plates (Maxisorb, Nunc, Denmark) were coated with 100 µL of 1µg/mL BSA in PBS overnight at 4°C. The plates were washed 4 times with PBS containing 0.5% Tween-20 between every step. The remaining binding sites were blocked with 100 µL 7% skimmed milk powder (Fluka). Serial 2-fold dilutions in 100 µL of PBS of serum

or saliva samples were applied in triplicates and incubated for 1 h at RT. The rabbit antibodies were detected with horseradish peroxidase conjugated goat-anti-rabbit IgG 1:10000 (v:v) or horseradish peroxidase conjugated goat-anti-rabbit IgA 1:500 (v:v). The 1,4-phenylenediamine substrate (Sigma) (100 µL) was allowed to react for 15 min. The reaction was stopped with 100 µL 1 M sulphuric acid and 10 min later the plates were examined in an ELISA reader at 492 nm as described earlier (Bollen *et al.,* 1995). The antibody titres were determined as the reciprocal of the highest dilution of saliva or serum (the optical density was measured at 492 nm) (Titertek Multiscan Plus MK II, Labsystems Finland), which generated a 2-fold higher colour signal than that of the negative samples.

The titres were converted to a base-2 logarithmic scale. The geometric means (GM) were calculated from $GM=\Sigma\ antilog_2/n$, where the numerator is the sum of the antilogarithms of all titre values (in log_2) and the denominator is the number of samples (Hoy, 1992).

Statistical analysis

Statistical evaluation of the results was done by one-way analysis of variance ANOVA using PRISM Software (Graph Pad Software, San Diego, CA, USA). The means of the IgA, IgG antibody titres were compared using paired, two-tailed *t*-test. All values were expressed as mean±standard deviation and were considered to be statistically significant at $P<0.05$.

RESULTS

Generally, immunospecific anti-BSA IgA antibodies in saliva were recorded in groups A (*Vinca mayor*), B (*Vitex agnus-castus*), D (platinum nanoparticles) and E ($Al[OH]_3$) of rabbits during the observation period, except for group F (mixture of seven plants) (Table 1). At day 56 of the last immunization, immunospecific anti-BSA IgA antibodies were detected in group C (*Aloe arborescens*). Furthermore, in groups D and E the IgA titres in saliva were significantly higher than those in other groups at day 56.

As shown (Table 2), the immunospecific anti-BSA IgG antibodies in serum were detected in all groups of rabbits examined throughout the observation period. In particular, the IgG titres of group F (mixture of 7 plants) were significantly reduced from day 14 to day 56 after the last immunization. Furthermore, in groups A, C, and F the IgG titres were significantly higher than those in other groups at day 14.

DISCUSSION

The usual response of the gastrointestinal tract to antigens is tolerance rather than immunity, as previously described (Chen *et al.,* 1995; Strobel and Mowat, 1998).). A combination of soluble antigen, boosting element and administration protocol was precisely selected. Our model of study is tightly composed and corresponds to the other studies where BSA was used as the antigen to provoke immune response in hens (Mayo *et al.,* 2003; Jonauskienė and Bižanov, 2008; Bižanov *et al.,* 2010, 2012). First, an antigenic capacity of BSA is enough to cause gentle immune

Table 1: Saliva IgA antibody response (log_2) in rabbits after the oral immunization with bovine serum albumin in combination with herb extract from *Vinca major*, *Vitex agnus-castus*, *Aloe arborescens*, platinum nanoparticles, aluminium hydroxide and the mixture of 7 plants.

Group	Days after the last immunization				
	0 d	14 d	28 d	42 d	56 d
Vinca major	2.7±0.6	6.5±0.1**	5.9±0.5**	5.0±0.4*	5.3±0.3*
Vitex agnus-castus	2.3±0.6	5.7±0.1*	5.7±0.2*	5.7±0.6*	5.3±0.3*
Aloe arborescens	2.7±0.6	4.0±0.7	3.7±0.2	3.6±0.1	6.0±0.1*
Platinum nanoparticles	2.3±0.6	5.8±0.7***	6.3±1.0*	6.0±0.6**	7.3±0.1**
$Al(OH)_3$	2.7±0.6	8.1±0.8*	7.6±0.8**	7.5±0.9**	8.1±0.5**
Mixture of 7 plants	2.7±0.6	3.3±0.6	3.3±0.6	3.8±0.7	3.3±0.6

Means with differing asterisks differ significantly from means at 0 d in the same row: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Table 2: Serum IgG antibody response (log_2) in rabbits after the oral immunization with bovine serum albumin in combination with herb extract from *Vinca major*, *Vitex agnus-castus*, *Aloe arborescens*, platinum nanoparticles, aluminium hydroxide and the mixture of seven plants.

	Days after the last immunization				
Group	0 d	14 d	28 d	42 d	56 d
Vinca major	4.6±0.6	12.3±0.3**	11.0±0.6***	11.2±0.3***	11.3±0.6**
Vitex agnus-castus	4.6±0.6	9.7±0.6***	9.6±0.3**	9.5±0.2**	9.5±0.2**
Aloe arborescens	4.6±0.6	13.3±0.6**	12.3±0.5**	12.3±0.6**	12.3±0.5**
Platinum nanoparticles	4.0±0.6	7.9±0.6*	8.3±0.6***	7.9±0.5**	8.6±0.4**
$Al(OH)_3$	4.9±0.6	7.9±0.6*	8.5±0.5*	8.5±0.6*	8.5±0.6**
Mixture of seven plants	3.9±0.6	10.6±0.6**	10.3±0.6**	9.1±0.5*	8.3±0.6**

Means with differing asterisks differ significantly from means at 0 d in the same row: *P<0.05, **P<0.01, ***P<0.001.

response (Klipper *et al.,* 2000), but it is usually combined with immunostimulator as a booster element. Second, the immunization mixture can be additionally enriched by emulsifying with an oil to provoke a prolonged enhancing effect (Hurn and Chantler, 1980; Masuda *et al.,* 2003). This 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 entry causes a reactivation of secretory as well as humoral components of the immune system.

We assessed the immunostimulatory capacity of extracts from *Vinca major*, *Vitex agnus-castus*, *Aloe arborescens*, in a mixture of 7 extracts, as well as aluminium hydroxide and platinum nanoparticles, under described conditions.

In this study, we have demonstrated that oral administration with BSA in combination with extracts from *Vinca major*, *Vitex agnus-castus*, *Aloe arborescens* and the polyherbal product containing 7 extracts from *Sambucus nigra*, *Primula versis*, *Pinus alba*, *Gentiana lutea*, *Cetraria islandica*, *Eucaliptus globulus* and *Citrus limon,* as well as aluminium hydroxide and platinum nanoparticles, could also prime the immune system for both secretory and systemic antibody responses. It is shown that the developed immune response can persist for longer period, even until 2 mo, after the first administration.

These data supplement previous reports about plant and mineral potency for the mammalian organism. Fortun-Lamothe and Drouet-Viard (2002) noted that biologically active constituents of plants as well as some trace elements are essential for the development and maintenance of humoral and cellular immunity. Vahedi *et al.,* (2011) described that feeding with *Aloe vera* extract stimulated increase of CD4+ lymphocyte in blood and in serum IgM and IgG antibodies over 21 d.

Using platinum salt-treated animals has shown cellular and humoral responses in guinea pigs (Liu, 1991) and in mice (Ban *et al.,* 2010). The ability of aluminium as an adjuvant to stimulate the production of IgG1 and IgG2a subclasses in mice was observed by Lindblad (2004).

On the one hand, polyherbal mixture containing extracts from *Panax ginseng*, *Poria cocos*, *Atractylodes macrocephala* and *Glycyrrhiza urales* induced weak response of IgA in chickens (Zhang *et al.,* 2012). On the other, the administration of extracts of a mixture of *Thujae summitates*, *Baptisiae tinctoriae*, *Echinaceae purpureae* and *Echinaceae pallidae* caused an enhancement of the antibody response to antigen (Bonidet *et al.,* 2002).

Our findings show that all extracts can be advised for immunostimulatory application as adjuvants in the rabbit model.

This work is a first report on the influence of aluminium hydroxide and platinum nanoparticles on secretory and humoral immune responses.

CONCLUSION

In our studies we have demonstrated that oral immunization with water-in-oil-emulsion based on mixture containing BSA in combination with the extracts from *Vinca major*, or *Vitex agnus-castus*, or *Aloe arborescens* and the polyherbal product containing 7 dry extracts from *Sambucus nigra*, *Primula versis*, *Pinus alba*, *Gentiana lutea*, *Cetraria islandica*,

Eucaliptus globulus, *Citrus limon,* as well as the aluminium hydroxide and platinum nanoparticle components mentioned above, could also prime the immune system for both secretory and humoral responses. In addition, the immunization mixture has an enhancing effect on the immune response in rabbits in the long term. It is important that the induction of antibody responses has taken place in the mucosa of the upper respiratory tract systemic booster in order to induce antibody responses to later repeated oral administrations.

Present findings show that some elements such as aluminium hydroxide and platinum nanoparticles may be used for immunomodulation *in vivo*.

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