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Antiviral and immunoregulatory role against PCV2 *in vivo* of Chinese herbal medicinal ingredients

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Abstract

Introduction: The aim of the research was to investigate the antiviral and immunoregulatory effects of saikosaponin A, saikosaponin D, Panax notoginseng saponins, notoginsenoside R1, and anemoside B4 saponins commonly found in Chinese herbal medicines. **Material and Methods:** control mice were challenged intramuscularly (im) with 0.2 mL of porcine circovirus 2 (PCV2) solution containing 10^7 $TCID_{50}$ of the virus/mL. Mice of high-, middle-, and low-dose saponin groups were initially challenged im with 0.2 mL of PCV2 solution and three days later treated intraperitoneally (ip) with one of five saponins at one of three doses (10, 5, or 1 mg/kg b.w.). In the drug control group, mice were dosed ip with 10 mg/kg b.w. of a given saponin, and mice in a blank control group were administered the same volume of normal saline. **Results:** The results revealed that the saponins could reduce the incidence and severity of PCV2-induced immunopathological damage, *e.g*. body temperature elevation, weight loss, anaemia, and internal organ swelling. In addition, it was seen that the saponins could affect the immunoglobulin levels and protein absorption. **Conclusion:** The data suggested that the saponins might effectively regulate immune responses.

Keywords: porcine circovirus, saponins, antivirus properties, immune system.

Introduction

Porcine circovirus 2 (PCV2) is the smallest known animal virus, belonging to the genus *Circovirus* in the *Circoviridae* family (4). PCV2 was demonstrated to be a causative agent of porcine circovirus-associated disease (PCVAD), which includes porcine multi-systemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), congenital tremor (CT), and reproductive failure (6, 12). Since its emergence in the early 1990s, PCVAD has continuously been a threat to the global swine industry, causing high economic losses (14, 28).

Vaccination is traditionally considered as the most effective method for preventing viral diseases (13, 16). However, the protection period given by the vaccine against disease is limited and the virus cannot be eradicated by vaccination (7). Furthermore, no effective vaccines are available for preventing multifactorial disease such as PCVAD (24). Therefore finding alternative effective measures to control the disease is an urgent need. Many Chinese herbal medicines can effectively suppress viral pathogens, in addition to eliminating fever and clearing toxins (26). They are also widely used to prevent or cure other non-viral infectious diseases, and show higher efficacy, lower toxicity, fewer side-effects, and lower residual levels than many commonly used drugs.

Saikosaponin A (SSA) and saikosaponin D (SSD) are major triterpenoid saponins derived from *Bupleurum falcatum* L. (*Umbelliferae*), commonly prescribed by Chinese and Japanese doctors for inflammatory and infectious diseases. These active components are reported to impart immunomodulatory, anti-inflammatory, anti-bacterial, anti-viral, and anti-cancer effects (15). Recently, it has been shown that SSD could exhibit an anti-proliferative effect in activated T-lymphocyte, in part *via* suppression of NF-κB, NF-AT, and AP-1 signalling (25).

Panax notoginseng saponins (PNS) are the major active components of notoginseng. They consist of >30 different types of saponins; among these, ginsenosides Rg1 and Rb1 are found at high levels. PNS exhibit anti-cancer activity and have been shown to be effective against a variety of malignancies including colorectal, lung, gastric, skin, prostate, and liver cancers (2). Notoginsenoside R1 (SR1) is a component unique to notoginseng (27). It has been shown to be a promising compound for protecting the heart from septic shock and to impart anti-inflammatory effects (19). Pulsatilla koreana Nakai, with anemoside B4 (AB4) as its main pharmacological effective compound, is known to have numerous biological effects, including hypoglycaemic, anti-tumour, neuroprotective, and anti-angiogenic activity (20).

In the present study, the effects of five saponins, namely SSA, SSD, PNS, SR1, and AB4, were evaluated as antiviral and immunoregulatory agents. The effects of each were assessed *via* measures of impact on physiological and biochemical blood indices following exposure to PCV2. The aim of the studies was to provide a theoretical basis for further research on saponins and development of new drugs for use as antiviral and immunoregulatory agents.

Material and Methods

Reagents. Pure SSA, SSD, PNS, SR1, and AB4 (each at 20 mg/vial; lot #110777, #110778, #110745, #110870, and #111766, respectively) were all purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). All ingredients were individually diluted to three concentrations (1, 5, and 10 mg/mL) with normal saline. The diluted solutions were then filtered through a 0.22-μm membrane and stored at 4ºC.

Virus. The strain of PCV2 was isolated from a suspected PMWS case (11) at the Zoonotic Prevention and Control Laboratory at Jiangsu Academy of Agricultural Sciences and propagated on PK-15 cells. The titre of the virus, determined by IFA and PAMs, was 10^7 $TCID_{50}$/mL.

Animals. Balb/c mice (male, 18–22 g, six weeks of age), pre-tested negative for PCV2, were purchased from the Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). A total of 66 completely healthy mice were used. All mice were housed in pathogen-free facilities, maintained at 20ºC with 50% relative humidity and 12-h light:12-h dark cycle. All mice had *ad libitum* access to filtered tap water. All protocols used here were approved by the Department of Science and Technology of Jiangsu Province (license number SYXK (SU) 2015-0005). All efforts were made to minimise suffering.

Experimental protocol. The mice were randomly assigned to 22 equal groups. Over the course of the post-treatment period, each group was housed separately in different isolation rooms with individual ventilation. In the PCV2 control group, each mouse was challenged intramuscularly (im) with 0.2 mL of PCV2 solution containing 10^7 $TCID_{50}$ of the virus/mL. In high-, middle-, and low-dose saponin groups, mice were initially challenged with 0.2 mL of the same PCV2 solution and three days later dosed intraperitoneally (ip) with one of five saponins at one of three doses (10, 5, or 1 mg/kg b.w.). In the drug control group, mice were treated ip with 10 mg/kg b.w. of a given saponin, while mice in the blank control group were administered the same volume of normal saline. The animal groups and experimental schedule used are shown in Table 1.

Determinations of physiological index. All animals were observed daily for clinical signs, and euthanised on day seven post-infection (dpi). Blood was collected from the retro-orbital plexus on the 7th dpi and serum was used for measures of IgG, IgM, albumin (ALB), globulin (GLO), total protein (TP), and alkaline phosphatase (ALP) using an AC-7020 automatic biochemical analyser (Hitachi, Japan). Blood collected into anti-coagulant coated tubes on the 7th dpi was used for the analyses of haemoglobin (HGB) levels and white blood cell (WBC), red blood cell (RBC), platelet (PLT), and lymphocyte (LY) counts using a GTR-6000 automatic blood analyser (Glett, China).

Statistical analyses. All data were expressed as means ±SD. A one-way analysis of variance (ANOVA) was used to determine the significance of differences between the PCV2 control group and the low-, middle-, and high-dose groups of each saponin, blank control group, and individual saponin control groups. All statistical analyses were performed using SPSS statistical software (v17.0, SPSS Inc., USA). When ANOVA results were significant, multiple comparisons of means were performed using a Dunnett's test analysis. Significant differences were assumed at $P < 0.05$).

Results

TP and ALB levels. Compared with the PCV2 control group, TP levels were significantly increased in high- and middle-dose SSA, SSD, and PNS groups, and in the high-dose SR1 and AB4 groups (Table 2). This effect was (maximally) induced by 19.6%, 19.6%, 21.6%, 20.8%, 25.5%, 17.3%, 23.2%, and 20.4% with high and middle doses of SSA, SSD, and PNS, and high doses of SR1 and AB4, respectively. The ALB level in the PCV2 control mice was significantly decreased (by 18.5%) to that in the blank control mice (Table 3). Compared with the PCV2 control group, ALB levels were significantly increased in the high- and middle-dose PNS groups (27.6% and 19.5%), and in the high-dose SR1 group (22.9%).

IgG and IgM levels. Compared with the PCV2 control group, IgG levels were significantly increased in the high-dose SSA, SSD, and AB4 groups (Table 4). This effect was (maximally) induced by 17.9%, 19.1%, and 17.1%, with high-dose SSA, SSD, and AB4,

respectively. No doses of the saponins had any impact on PCV2-induced IgM levels (Table 5).

WBC and RBC counts. As expected, WBC counts in PCV2 control mice decreased significantly (by 36.7%) *versus* blank control mouse values (Table 6). Compared with PCV2 controls, WBC counts were significantly increased in high- and middle-dose SSA mice, and in high-dose SSD, PNS, and AB4 mice. WBC levels were maximally induced by 63.3%, 46.2%, 54.7%, 50.7%, and 70.9%, with high and middle doses of SSA, and high doses of SSD, PNS, and AB4, respectively. The RBC counts decreased by 18.4% in PCV2-only mice (Table 7). Compared with PCV2 control values, RBC counts increased significantly by 31.7%, 19.2%, 30.0%, and 21.8% in high- and middle-dose PNS and SR1 mice, respectively.

Table 1. Animal groups and experimental schedule used

Groups	0 h by intramuscularly		3 h intraperitoneally	
	reagent	dose	reagent	dose
PCV2 control	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	-	-
SSA high-dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	SSA	10 mg/kg
SSA middle-dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	SSA	5 mg/kg
SSA low-dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	SSA	1 mg/kg
SSD high-dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	SSD	10 mg/kg
SSD middle-dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	SSD	5 mg/kg
SSD low -dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	SSD	1 mg/kg
PNS high-dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	PNS	10 mg/kg
PNS middle-dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	PNS	5 mg/kg
PNS low -dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	PNS	1 mg/kg
SR1 high-dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	SR1	10 mg/kg
SR1 middle-dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	SR1	5 mg/kg
SR1 low -dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	SR1	1 mg/kg
AB4 high-dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	AB4	10 mg/kg
AB4 middle-dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	AB4	5 mg/kg
AB4 low -dose	PCV2	10^7 $TCID_{50}$ virus/mL with 0.2 mL	AB4	1 mg/kg
SSA control	-	-	SSA	10 mg/kg
SSD control	-	-	SSD	10 mg/kg
PNS control	-	-	PNS	10 mg/kg
SR1 control	-	-	SR1	10 mg/kg
AB4 control	-	-	AB4	10 mg/kg
Blank control	normal saline	the same volume	-	-

Table 2. TP contents (g/L) in blood sera of each group

Groups	Ingredient concentration			Drug control 10 mg/kg	PCV2 control 10^5TCID50	Blank control -
	10 mg/kg	5 mg/kg	1 mg/kg			
SSA	58.67 ± 6.21*	58.67 ± 7.44*	54.4 ± 4.77	56.0 ± .8		
SSD	59.67 ± 1.31**	59.3 ± 4.18**	54.43 ± 4.8	55.4 ± 6.54		
PNS	61.57 ± 4.3**	57.57 ± 2.35*	53.2 ± 5.02	54.87 ± 3.0	28.4 ± 1.45	52.07 ± 1.91
SR1	60.43 ± 8.52**	55.8 ± 1.55	54.4 ± 5.0	54.13 ± 3.89		
AB4	59.07 ± 3.1*	54.33 ± 5.19	51.0 ± 0.79	51.93 ± 2.61		

Data shown are mean (± SD) g/L
Value significantly different from PCV2 control group at * $P < 0.05$ or ** $P < 0.01$

Table 3. ALB contents (g/L) in blood sera of each group

Groups	Ingredient concentration			Drug control 10 mg/kg	PCV2 control 10^5TCID50	Blank control -
	10 mg/kg	5 mg/kg	1 mg/kg			
SSA	31.97 ± 4.06	32.47 ± 4.99	29.8 ± 2.88	35.37 ± 2.22		
SSD	32.57 ± 2.15	32.67 ± 3.12	30.6 ± 3.29	33.4 ± 4.16		
PNS	36.23 ± 2.4**	33.93 ± 1.46*	31.27 ± 2.18	36.03 ± 2.3	4.92 ± 0.35	34.83 ± 1.58**
SR1	34.90 ± 3.73**	32.60 ± 3.03	31.10 ± 3.04	35.13 ± 1.25		
AB4	33.00 ± 2.55	30.83 ± 1.7	28.77 ± 2.59	33.20 ± 1.78		

Data shown are means (± SD) g/L
Value significantly different from PCV2 control group at * $P < 0.05$ or ** $P < 0.01$

Table 4. IgG contents (g/L) in blood sera of each group

Groups	Ingredient concentration			Drug control 10 mg/kg	PCV2 control 10^5TCID50	Blank control -
	10 mg/kg	5 mg/kg	1 mg/kg			
SSA	5.8 ± 0.23*	5.35 ± 0.73	5.02 ± 0.28	4.81 ± 0.47		
SSD	5.86 ± 0.28*	5.6 ± 0.57	5.23 ± 0.29	4.96 ± 0.27		
PNS	5.53 ± 0.72	5.12 ± 0.48	4.70 ± 0.57	4.54 ± 0.48	4.92 ± 0.35	4.18 ± 0.38
SR1	5.34 ± 0.78	5.23 ± 0.61	4.57 ± 0.29	4.68 ± 0.41		
AB4	5.76 ± 0.4*	5.39 ± 0.67	4.88 ± 0.69	4.64 ± 0.51		

Data shown are mean (± SD) g/L
Value significantly different from PCV2 control group at * $P < 0.05$

Table 5. IgM contents (g/L) in blood sera of each group

Groups	Ingredient concentration			Drug control 10 mg/kg	PCV2 control 10^5TCID50	Blank control -
	10 mg/kg	5 mg/kg	1 mg/kg			
SSA	0.93 ± 0.13	0.84 ± 0.24	0.79 ± 0.21	0.87 ± 0.1		
SSD	1.0 ± 0.18	0.85 ± 0.14	0.76 ± 0.14	0.9 ± 0.14		
PNS	0.88 ± 0.18	0.82 ± 0.11	0.77 ± 0.13	0.75 ± 0.1	0.82 ± 0.09	0.67 ± 0.11
SR1	0.87 ± 0.13	0.8 ± 0.19	0.83 ± 0.18	0.85 ± 0.15		
AB4	0.96 ± 0.11	0.91 ± 0.25	0.85 ± 0.05	0.83 ± 0.12		

Data shown are mean (± SD) g/L
Value significantly different from PCV2 control group at * P < 0.05

Table 6. WBC contents (10^9/L) in anticoagulated blood of each group

Groups	Ingredient concentration			Drug control 10 mg/kg	PCV2 control 10^5TCID50	Blank control -
	10 mg/kg	5 mg/kg	1 mg/kg			
SSA	18.13 ± 3.88**	16.23 ± 2.3*	13.60 ± 1.35	18.77 ± 2.35		
SSD	17.17 ± 1.4*	15.03 ± 1.42	15.23 ± 2.35	17.50 ± 1.57		
PNS	16.73 ± 4.7*	14.97 ± 3.1	13.33 ± 4.49	16.93 ± 4.62	11.10 ± 1.93	17.53 ± 1.7**
SR1	15.27 ± 2.87	14.97 ± 3.26	14.40 ± 2.35	16.90 ± 3.12		
AB4	18.97 ± 1.67**	15.30 ± 2.46	15.20 ± 2.74	19.57 ± 2.7		

Data shown are mean (± SD) 10^9/L
Value significantly different from PCV2 control group at * P < 0.05 or ** P < 0.01

Table 7. RBC contents (10^{12}/L) in anticoagulated blood of each group

Groups	Ingredient concentration			Drug control 10 mg/kg	PCV2 control 10^5TCID50	Blank control -
	10 mg/kg	5 mg/kg	1 mg/kg			
SSA	5.68 ± 0.64	5.79 ± 0.19	5.67 ± 0.55	5.71 ± 0.32		
SSD	5.69 ± 0.48	5.67 ± 0.49	5.71 ± 0.56	6.14 ± 0.70		
PNS	6.53 ± 0.58**	5.91 ± 0.68*	5.70 ± 0.63	6.61 ± 0.54	4.96 ± 0.49	6.08 ± 0.64*
SR1	6.45 ± 0.72**	6.05 ± 0.63*	5.65 ± 0.92	6.04 ± 0.37		
AB4	5.81 ± 0.55	5.75 ± 0.24	5.46 ± 0.76	5.69 ± 0.29		

Data shown are mean (± SD) 10^{12}/L
Value significantly different from PCV2 control group at * P < 0.05 or ** P < 0.01

Table 8. HGB contents (g/L) in anticoagulated blood of each group

Groups	Ingredient concentration			Drug control 10 mg/kg	PCV2 control 10^5TCID50	Blank control -
	10 mg/kg	5 mg/kg	1 mg/kg			
SSA	120.3 ± 13.32**	108.3 ± 7.77	109.3 ± 9.87	115.7 ± 10.26		
SSD	111.0 ± 12.77	107.7 ± 11.06	104.0 ± 9.0	124.3 ± 13.01		
PNS	125.3 ± 8.02**	118.7 ± 6.51*	109.0 ± 8.54	121.0 ± 12.53	97.7 ± 6.11	123.7 ± 11.93**
SR1	126.7 ± 12.66**	122.0 ± 8.54**	112.7 ± 9.5	127.3 ± 10.41		
AB4	113.0 ± 9.64	105.3 ± .64	97.0 ± 8.19	113.0 ± 11.14		

Data shown are mean (± SD) g/L
Value significantly different from PCV2 control group at * P < 0.05 or ** P < 0.01

Table 9. PLT contents (10^9/L) in anticoagulated blood of each group

Groups	Ingredient concentration			Drug control 10 mg/kg	PCV2 control 10^5TCID50	Blank control -
	10 mg/kg	5 mg/kg	1 mg/kg			
SSA	246.7 ± 35.64	249.7 ± 42.53	225.7 ± 43.84	220.0 ± 41.22		
SSD	274.3 ± 41.5	267.0 ± 34.4	263.0 ± 36.59	261.3 ± 34.12		
PNS	249.0 ± 24.98	265.3 ± 16.65	267.3 ± 46.92	249.7 ± 47.44	237.3 ± 46.18	250.3 ± 46.05
SR1	264.0 ± 49.73	252.0 ± 18.03	261.7 ± 34.78	281.7 ± 37.5		
AB4	257.7 ± 41.07	237.7 ± 29.37	248.3 ± 45.49	270.3 ± 33.01		

Data shown are mean (± SD) 10^9/L
Value significantly different from PCV2 control group at * P < 0.05

HGB and PLT levels. The HGB levels decreased by 21.0% (relative to control) in the PCV2 control group mice (Table 8). Compared with PCV2 control values, HGB levels were significantly increased, rising by 28.2%, 21.5%, 29.7%, 24.9%, and 23.1%, in high- and middle-dose PNS and SR1 and high-dose SSA mice respectively. No doses of the saponins had any impact on PCV2-induced PLT levels (Table 9).

Discussion

Chinese herbal medicine can regulate the balance of cellular functions and improve the physiological conditions of the organism in many ways. Accordingly, many studies have recently focused on the use of Chinese herbal medicines to treat or prevent PCV2-induced health disorders (23). Existing data show that

PCV2 induces pathological effects which can cause an imbalance in a variety of protein levels and cell numbers (5, 17, 18). The experiments here suggested that each of the five test saponins could reduce the incidence and severity of some aspects of virus-associated pathologies, like the protein absorption barrier, immune suppression, and anaemia in mice. The present results also suggest that in general, saponins might be used as part of a therapeutic immunoenhancement regimen.

The serum TP value, to a certain extent, represents the level of proteins in the diet and degree of protein digestion and absorption (8). In the present study, it was seen that TP levels were significantly promoted by all tested saponins, with SSD having the greatest effect. Albumin, which can be used to repair tissue and provide energy, was shown to be significantly induced by PNS and SR1 in a dose-related manner. The TP consists of albumin and globulin. Panax pseudo-ginseng saponins could significantly increase the level of albumin to repair liver damage, while bupleuri saponins could significantly improve the level of globulin to enhance immune function. They all increase the total protein content, which promoted the absorption and utilisation of nutrients, but there is no improvement in the proportion of albumin to globulin.

IgG shows the highest concentration in the serum of animals. It can exhibit antibacterial, antiviral, and antitoxin immunological activity (10). At the same time, IgG was the main antibody in the serological diagnosis and monitoring of the vaccine (1). We found that the level of IgG was significantly increased by SSD. This could represent a mechanism by which saponins could sometimes be used in the treatment of immune disorders. It is known that IgG and IgM are important immunoglobulins in humoral responses. SSA and SSD could improve the neutralisation effect of the immunoglobulins against the virus (3, 29). Because of the low content of IgA in serum, we did not detect the correlation value.

During infection-related stress, there is a typical decrease in the number of WBC and RBC. The present study showed that the WBC count was significantly increased (relative to levels in infected control hosts) by all the saponins except SR1. Many studies have revealed that neutrophil number down-regulation is one of the basic characteristics of PCV2 infection. The current study confirmed this phenomenon. It is clear that down-regulation of WBC may cause immunosuppression. The effects of WBC are diverse but mainly comprise anti-inflammatory activity, mediating the negative feedback of T_H1 and T_H2 immune responses. Previous findings have confirmed that RBC also has an immunoregulatory function. The ability of PNS and SR1 to increase RBC number after PCV2 exposure was much higher than that noted in their respective control groups.

Saponins possess the ability to clear toxic materials, alleviate pain, and improve blood circulation (21, 22). In the present study, the amount of HGB was dramatically down-regulated in response to PCV2. Such findings are in line with those of other studies. During a PCV2 infection, down-regulated HGB production may result in small cell anaemia. In the current study there was up-regulation of HGB by PNS or SR1 after direct stimulation with PCV2. Ongoing studies in our laboratories will need to ascertain the modulated pathways of saponins tested in immune and pathological physiology.

The five saponins may exert some therapeutic effects on the immune and circulatory systems and counter immunomodulatory effects and related syndromes induced by viral infection, at least in part by modulating the content of immunoglobulin and HGB. In addition, it was seen that PCV2 could affect the temperature and weight of tested mice. The previous study *in vitro* showed that production of IL-4, IL-10, IL-2, and IFN was also specifically affected when target cells had already been stimulated with PCV2 (9). These findings warrant saponins being further explored for their antiviral activity in clinical settings. Future studies will elaborate the specific way each of the tested saponins acts against the virus.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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