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Research article

EFFECTS OF HIGH MOLECULAR WEIGHT POLY -γ-GLUTAMIC ACID ON PIGS WITH PORCINE PREPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) INFECTION

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Bacillus subtilis sups. chungkookjang produces a higher molecular mass poly-γ-glutamic acid (γ-PGA). Recently, previous studies have demonstrated immune stimulation and an antitumor effect of the high molecular mass γ-PGA using various mouse models although these effects have not been shown in other species of animals. Therefore, the current study was conducted to determine the effect of γ-PGA in pigs with and without PRRSV infection. PRRS-negative pigs were intramuscularly injected with 1, 3, or 5 ml of 20 mg/m*l* γ-PGA, and one group of pigs served as a non-treatment (NT) group. All groups treated with γ-PGA had significantly higher weight gains, and pigs treated with 5 ml of γ-PGA exhibited higher tumor necrosis factor (TNF)-α, interferon (IFN)-α and IFN-β expression levels compared with the NT group. According to the preliminary results, an animal challenge study was conducted with a highly virulent PRRSV strain, MN184, along with γ-PGA treatment at different time points. Pigs treated with γ-PGA had lower levels of viral loads in the sera and in lungs and gained significantly more weight ($p<0.05$) compared with the NT group after being challenged with MN184. Moreover, γ-PGA-treatment groups had higher levels of neutralizing antibodies and cytokines related to proinflammatory, humoral and cell-mediated responses than the control group after the PRRSV challenge. Therefore, it was concluded that γ-PGA induces higher levels of immune responses and increases resistance to PRRSV infection in pigs.

Key words: antiviral activity, immune stimulation, neutralizing antibody, poly-γ-glutamic acid, PRRSV

INTRODUCTION

Poly-γ-glutamic acid (γ-PGA) is a natural biomaterial that is produced from various strains of *Bacillus subtilis* through a process of fermentation [1]. *Bacillus subtilis chungkookjang*, a strain used for the production of a transitional Korean fermented

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seasoning soybean food, has been reported to uniquely produce a higher molecular weight γ-PGA (HM-γ-PGA, >2,000 kDa) than that of *Bacillus subtilis natto* (10-1,000 kDa) [1-4]. γ-PGA is an anionic polymer and a naturally occurring homo-polyamide that is made of D- and L-glutamic acid units connected by amide bonds between α-amino and γ-carboxylic acid groups. γ-PGA is water soluble, biodegradable, edible and non-toxic to humans and the environment. Recent studies have shown that the high molecular mass γ-PGA from *Bacillus subtilis* possesses functional and immune activation effects. Thus, in the past few years, potential applications of γ-PGA and its derivatives have been of interest as biomaterials in a wide range of industrial fields, such as food, cosmetics/skin care, bone care, medicine and water treatment [4-6]. Previous studies have demonstrated that HM-γ-PGA exhibited significant antitumor activity in mice bearing major histocompatibility (MHC) class 1-deficient tumors. HM-γ-PGA is potentially a good candidate as an antitumor immune mediator via the activation of natural killer (NK) cells [7]. Additionally, previous studies have demonstrated that treatment with HM-γ-PGA initiated the innate immune responses via Toll-like receptor (TLR) 4 signaling in mice, and oral administration of HM-γ-PGA induced TLR4-dependent antitumor effects in a mouse tumor model *in vivo* [8]. Previous studies have also demonstrated that HM-γ-PGA had antiviral activity. HM-γ-PGA exerts significant antiviral activity that can inhibit Newcastle disease virus infection by stimulating an antiviral state on RAW 264.7 cells [9]. Additionally, previous studies have shown that treatment with influenza inhibitors (polyvalent sialidase inhibitors) on the γ-PGA backbone may have antiviral activity against Influenza A virus [10].

Porcine reproductive and respiratory syndrome virus (PRRSV) is a cause of major economic loss to the pig industry worldwide. PRRSV infection causes reproductive problems in pregnant sows and respiratory syndrome in pigs of all ages [11-13]. PRRSV infection has spread to many countries and causes significant economic losses on swine farms. PRRSV was first isolated in Europe in 1991 and in North America in 1992 [14,15]. To control PRRSV in the field, management strategies, such as herd depopulation, repopulation, herd closure, bio-security, and testing and removal, have been attempted. Vaccinations are also widely used for most of the pig populations. Both killed virus (KV) vaccines and modified live virus (MLV) vaccines are commercially available and commonly used. However, inactivated and MLV vaccines are ineffective in providing sufficient protective immunity because PRRSV exists as various genotypes. There are also concerns about the reversion of vaccine strains to virulent genotypes [16]. As a result, there has been a high demand for the development of alternative efficient methods for disease control.

The objectives of the present study are to investigate the immunological effects and the therapeutic and preventive efficacies of HM-γ-PGA produced by *Bacillus subtilis sups. chungkookjang* in PRRSV-negative pigs and pigs challenged with PRRSV strain MN184.

MATERIALS AND METHODS

Preparation of HM-γ-PGA

HM-γ-PGA produced by *Bacillus subtilis* subsp. *chungkookjang* was prepared and provided by BioLeaders Corporation (Daejeon, Korea) in 0.85% sterile NaCl solution, as described in previous studies [3,7,9]. Briefly, the culture broth of *B. subtilis* subsp. *chungkookjang* was collected and mixed with a 3X volume of ethanol. The precipitate was lyophilized and reconstituted in 10 mM Tris-HCl buffer (pH 7.5), treated with proteinase K, and dialyzed in distilled water. Next, the γ-PGA was purified by anion-exchange chromatography and dialyzed using a Sep-Pak Plus Waters Accell Plus QMA cartridge (Millipore, Darmstadt, Germany) equilibrated with distilled water. Next, the cartridge column charged with γ-PGA was stepwise developed with NaCl solutions from 0.1 to 1.0 M. By estimating the concentration of glutamate in hydrolyzed γ-PGA using an amino acid analyzer, the content of γ-PGA was calculated by the following formula: content of γ-PGA (%) = (amount of glutamate/amount of sample) × (A/B) × 100, with A = 129 (molecular mass of γ-glutamyl residue in γ-PGA) and B = 147 (molecular mass of glutamate). The number and weight-average molecular masses (Mn and Mw, respectively) along with the polydispersity (Mw/Mn) of γ-PGA molecules were measured by gel permeation chromatography using a GMPWXL column (Viscotek, TX, USA) and an LR125 Laser Refractometer (Viscotek, TX, USA). Polyacrylamide standards (American Polymer Standard, OH, USA) were used to construct a calibration curve, and the polydispersity of HM-γ-PGA was measured. The content of HM-γ-PGA was increased to >99%, and polydispersity was decreased after anion-exchange chromatography. To obtain thoroughly solubilized γ-PGA, the pH was adjusted to 7.0 by adding 5N sodium hydroxide (NaOH) solution to the acid form of PGA.

Viruses and cells

A North America (type II) PRRSV strain, MN184 (GenBank # EF488739), was used in the study. The designation, origin, and genetic and biological characteristics of the virus have been described in detail previously [17]. The virus was propagated in MARC-145 cells, an African Green Monkey kidney cell line highly permissive to PRRSV infection. MARC-145 cells were maintained in RPMI-1640 medium supplemented with heat-inactivated 10% fetal bovine serum (FBS, Gibco, CA, USA), 2 mM L-glutamine, and 100X antibiotic-antimycotic solution [Anti-anti, Invitrogen; 1X solution contains 100 IU/m/penicillin, 100 μg/m/streptomycin, and 0.25 μg/m/Fungizone® (amphotericin B)] at 37°C in a humidified 5% CO_2 atmosphere.

Animal experiments

Two consecutive animal experiments were conducted. Experiment 1 was conducted to determine the optimal HM-γ-PGA dose required to stimulate immunity without

causing adverse effects in pigs. Twenty 4-week-old pigs were purchased from a pig farm that was negative for PRRSV, porcine circovirus type 2, swine influenza virus and *Mycoplasma hyopneumoniae*. The pigs were randomly divided into 4 groups, and 3 groups were injected 2 times with 1, 3 or 5 m*l* of HM-γ-PGA, respectively, at 7-day intervals. One group was kept as a non-treatment (NT) group and given 5 ml of phosphate-buffered saline (PBS). Serum and peripheral blood mononuclear cells (PBMCs) were collected from all pigs before the experiment and at 0, 3 and 7 days after the second HM-γ-PGA injection. Body weight was measured every week to determine the average daily weight gain (ADWG) during the experiment. The second experiment was conducted to evaluate the therapeutic effects of HM-γ-PGA on pigs infected with MN184 at the titer of 10^3 $TCID_{50}$/ml, a moderately virulent PRRSV strain. Twelve 4-week-old pigs were also purchased and randomly divided into 4 groups. Then, all pigs were challenged with MN184, and pigs in the three groups were treated with 5 m*l* HM-γ-PGA at 0, 3 or 7 days post-challenge (dpc). One group was kept as a challenge control group and was given 5 ml of PBS. Serum and PBMCs were collected from all of the pigs on a weekly basis. Weight was measured to determine the ADWG during the study. The animal experimental protocol was approved by the Chonbuk National University Institutional Animal Care and Use Committee (Approved Number: 2012-0025).

PRRSV real-time RT PCR

The virus levels in the sera were measured by real-time reverse transcription-polymerase chain reaction (RT-PCR) using TaqMan® chemistry. Primers and minor groove binder (MGB) fluorescent probes specific to a conserved region of open reading frame 7 (ORF7) were designed using Primer Express® software V 3.0 (Applied Biosystems, CA, USA). The primer and probe sequences were: Forward Primer: TGTCAGATTCAGGGAGRATAAGTTAC; Probe: TTTTGCACCACMGCCAGCCC; Reverse Primer: ATCARGCGCACAGTRTGATGC. Real-time RT-PCR was performed using an AgPath-IDTM One-Step RT-PCR Kit (Ambion, TX, USA) with a 7500 Fast Real-time PCR system (Applied Biosystems, CA, USA). Cycling conditions were as follows: (a) reverse transcription for 10 min at 45°C; (b) a 10 min activation step at 95°C; and (c) 40 cycles of 15 sec at 95°C and 45 sec at 60°C. Samples with threshold cycle (Ct) counts of 35 cycles or less were considered positive. A standard curve previously made of known virus titers was used to calculate the amount of PRRSV in each sample by converting the Ct value to virus titer (50% tissue culture infectious dose [$TCID_{50}$]/m*l*).

Purification of PBMCs

Blood from all of the pigs was collected 21 days after the viral challenge, and porcine PBMCs were isolated using the density gradient method in Histopaque®-1077 solution (Sigma, St. Louis, MO, USA) from 3 ml of blood samples collected in lithium-heparin-containing vacutainers according to the manufacturer's instructions. The blood

samples were briefly stratified on Histopaque®-1077 solution at a ratio of 1:1 (blood: Histopaque) and were centrifuged at 400 x g for 30 min. The purified PBMCs were collected and washed twice with sterile PBS (pH 7.0) supplemented with 1% FBS and were re-suspended in 0.5 ml of sterile PBS. To evaluate cell viability and number, the cells were diluted 100 times in PBS, mixed with 0.4% Trypan Blue at a 1:1 ratio and counted using a Countess™ Automated Cell Counter (Invitrogen, CA, USA).

Determination of cytokine expression

Cells were diluted to 5 x 10^6 cells/m*l* in RPMI-1640 medium as described above, and cells were seeded in 24-well plates (1 ml of cells per well) (BD Falcon, Franklin Lakes, NJ, USA). Cells were then infected with a multiplicity of infection (MOI) of 0.1 of MN184 and were incubated at 37°C in a humidified 5% CO_2 incubator. Cells were harvested 72 h after stimulation, and cellular RNA was extracted using a GeneAll® Hybrid-R™ kit (GeneAll Biotechnology, Seoul, South Korea) following the manufacturer's instructions. RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Real-time PCR was then performed on a 7500 Fast Real-time PCR system (Applied Biosystems, CA, USA) using various cytokine-specific primers (Table 1). The 10 μl of 2X Power SYBR® Green (Applied Biosystems, Foster city, CA), 2 μl of cDNA and 0.5 μl of each forward primer (10 pM/μl) and reverse primer (10 pM/μl) were used for PCR amplification. All of the samples were tested in duplicate, and the cycling conditions were as follows: (a) holding for 10 min at 95°C; (b) 40 cycles of 15 sec at 95°C and 1 min at 60°C; and (c) melt curve stage for 15 sec at 95°C, 1 min at 60°C, 15 sec at 95°C and 15 sec at 60°C. Relative quantities of cytokine mRNA in infected and non-infected cells were normalized to β-actin mRNA, and the relative amounts were determined using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primers for measuring mRNA expression levels of various cytokines

Genes	Forward Primer (5'- 3')	Reverse Primer (5'- 3')	*Accession /Reference
β-Actin	GCGGGACATCAAGGAGAAG	AGGAAGGAGGGCTGGAAGAG	U07786
TNF-α	TTATTCAGGAGGGCGAGGT	AGCAAAAGGAGGCACAGAGG	NM_214022
IFN-α	TCTCATGCACCAGAGCCA	CCTGGACCACAGAAGGGA	Loving et al. (2006)
IFN-β	AGTGCATCCTCCAAATCGCT	GCTCATGGAAAGAGCTGTGGT	de Los Santos et al. (2006)
IL-2	TGCACTAACCCTTGCACTCA	CCTGCTTGGGCATGTAAAAT	X56750
IL-4	TTGCTGCCCCAGAGAACAC	CATAATCGTCTTTAGCCTTTCC	NM_214123
IL-6	CCACCAGGAACGAAAGAGAG	AGGCAGTAGCCATCACCAGA	NM_214399
IL-10	TGACGATGAAGATGAGGAAGAA	GAACCTTGGAGCAGATTTTGA	NM_214041
IFN-γ	GACTTTGTGTTTTTCTGGCTCTTAC	TTTTGTCACTCTCCTCTTTCCA	NM_213948

Serum-virus neutralization assay

The serum-virus neutralization (SVN) assay was performed to evaluate SVN antibody titers induced by PRRSV after challenge using the fluorescent focus neutralization (FFN) method. The SVN assay was performed in MARC-145 cells as described previously [18,19], with some modifications. Briefly, the serum samples were heat-inactivated for 1 h at 56°C in a pre-heated water bath before starting the SVN assay. A ten-point, two-fold serial dilution of each serum sample was performed with RPMI-1640 growth medium in 96-well plates (BD, Falcon), and a sample volume of 100 µl was maintained for each dilution. An equal volume (100 µl) of MN184 at a titer of 10^3 $TCID_{50}$/ml was mixed with the diluted serum in each well, and the serum-virus mixture was then incubated for 1 h at 37°C. After incubation, the serum-virus mixture of each dilution was transferred onto MARC-145 cells in 96-well plates that had been prepared 48 h earlier. These mixtures were then incubated for 1 h under the same conditions described above. After incubation, the serum-virus mixture was discarded, and cells were replenished with 200 µl of RPMI-1640 growth medium and incubated for 20 h under the same conditions. After 20 h incubation, the plate containing cells was fixed with 80% cold aqueous acetone solution for approximately 5 min at -20°C and then air-dried at room temperature (RT). Fixed cells were incubated for 1 h with PRRSV-specific monoclonal antibody (Anti-PRRSV NC Monoclonal Antibody 4A.5, Jeno Biotech Inc., Chuncheon, South Korea) conjugated with fluorescein isothiocyanate (FITC) (Goat anti-Mouse IgG(H+L) FITC conjugated, Bethyl Laboratories, Inc., TX, USA). Cells were washed three times with PBS and 0.05% Tween® 20 (Tween® 20, Promega, WI, USA), and the number of virus-specific fluorescent foci in each well was counted under a fluorescence microscope (Nikon, Tokyo, Japan). The SVN antibody titer against each virus was calculated and expressed as the reciprocal of the highest dilution in which 90% or higher reduction in the number of fluorescent focus units (FFUs) was observed compared to the wells of the respective virus back titration.

Pathological evaluation

All pigs were humanly euthanized 4 weeks after PRRSV infection and were subjected to necropsy. The lungs were collected in 10% neutral buffered formalin and routinely processed for microscopic evaluation. Microscopic lesions were evaluated in a blind fashion and scored 0 (normal) to 4 (severe) for lesion severity.

Complete blood count (CBC) test

Whole blood samples were collected at 0, 3 and 7 days from all of the groups. The following biochemical parameters were determined using an automatic cell counter (MEK 6450K, Nihon Kohden, Tokyo, Japan) : white blood cells (WBCs) (*mcl*), red blood cells (RBCs) (*mcl*), hemoglobin (HGB) (g/d*l*), hematocrit (HCT) (%), mean corpuscular volume (MCV) (*fl*), mean corpuscular hemoglobin (MCH) (pg), mean corpuscular hemoglobin concentration (MCHC) (g/d*l*), and platelet count (PLT) (*mcl*).

Statistical analysis

All statistical analyses were conducted using SSPS (SAS Institute Inc., NC, USA). The viremia levels in the pigs were analyzed by calculating the area under the curve (AUC) and performing repeated measures ANOVA to define the overall differences; pairwise comparisons between viruses or groups were conducted using Dunnett's multiple comparison test. In addition, for non-parametric analyses, the Mann Whitney test was applied to determine differences in ADWG, lung viral loads and SVN titers of the pigs among the groups.

RESULTS

The effects of various doses of PGA injection in PRRS-negative pig

To determine innate immunity with different doses of HM-γ-PGA, expression levels of cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-α and IFN-β were examined at 0, 3 and 7 days after the 2nd dose of HM-γ-PGA injection. Slightly higher levels of TNF-α were observed in pigs injected with 5 ml HM-γ-PGA at7 days after HM-γ-PGA administration; however, the differences were not significant between groups. The IFN-α levels were similar in the group of pigs injected with HM-γ-PGA compared with the NT groups. Significantly higher IFN-β levels were observed in pigs injected with 5 m*l* of HM-γ-PGA at 7 days after HM-γ-PGA administration compared with the NT group (Fig. 1). Consistent with these results, all of the pigs treated with different doses of HM-γ-PGA had significantly higher weight gain compared with the pigs in NT group, although no differences were found among the HM-γ-PGA treatment groups (Table 2).

Table 2. Average daily weight gain (ADWG) in PRRS-free pigs after γ-PGA inoculation

Group	Days after challenge					ADWG (kg)	Group average (p-value)*
	0	7	14	21	28		
NT	5.4	6.8	11.1	13.0	14.0	0.38	0.37 ± 0.02
	5.2	5.9	9.3	11.7	14.0	0.38	
	5.4	5.9	9.0	12.2	13.3	0.34	
1 ml γ-PGA	5.7	7.5	10.7	15.0	16.0	0.45	0.47 ± 0.03 (0.005)
	4.3	7.5	9.4	14.1	14.6	0.45	
	6.2	9.0	11.9	16.0	17.7	0.50	
3 ml γ-PGA	6.6	8.9	12.0	15.7	17.2	0.46	0.52 ± 0.10 (0.028)
	6.1	8.3	12.8	16.4	16.9	0.47	
	6.6	10.6	16.3	19.3	21.2	0.63	
5 ml γ-PGA	6.5	7.2	11.5	12.4	15.7	0.40	0.44 ± 0.04 (0.022)
	6.3	8.4	12.1	13.8	17.1	0.47	
	7.2	8.0	12.4	12.6	17.2	0.43	

* Comparison with the NT group

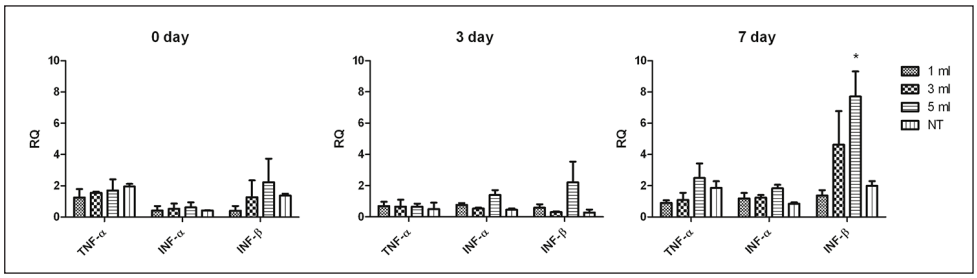

Fig. 1. Stimulation of innate immunity after PGA inoculation at various doses. The asterisk represents a value significantly different ($p<0.05$) from that in the NT group

HM-γ-PGA increased pig resistance to PRRSV infection

ADWG and viremia levels were measured in pig groups treated with 5 m/ HM-γ-PGA at 0 (PGA 0d), 3 (PGA 3d) and 7 (PGA 7d) dpc with PRRSV MN184 and were compared with levels in the virus challenge control (CC) group without HM-γ-PGA treatment. All groups treated with HM-γ-PGA had higher ADWG compared with pigs in the CC groups after challenge with PRRSV, strain MN184. Significantly higher ADWG was observed in the group with HM-γ-PGA treatment 7 dpc (PGA 7d) than in the CC group ($p<0.05$) (Table 3). The viremia levels detected in all of the pig groups treated with HM-γ-PGA were also significantly lower than those of the CC group based on repeated measures ANOVA and the calculation of the area under the viremia curve (Fig. 2). The levels of viral loads and microscopic lesion scores in lungs from the groups treated with HM-γ-PGA were generally lower than those from the CC group, but they were not significantly different (Fig. 3).

Table 3. Average daily weight gains in pigs infected with MN184 after PGA inoculation

Group	Days after challenge					ADWG	Group average (p-value)*
	0d	7d	14d	21d	28d		
Challenge	8	10	11	10	9	0.04	0.12 ± 0.07
	9	12	11	12	13	0.14	
	8	11	11.5	11	13	0.17	
PGA 0d	8	12	12	14	19	0.39	0.23 ± 0.14 (0.158)
	9	10	12	12	13	0.14	
	11	11	13	14	15	0.14	
PGA 3d	10	11	10	15	22	0.42	0.31 ± 0.15 (0.059)
	8	9	13	15.5	18	0.35	
	8	10	10	11	12	0.14	
PGA 7d	8	10	8	15	22	0.50	0.35 ± 0.14 (0.034)
	6.5	9	9	12	15	0.30	
	7	9	10	12	13.5	0.23	

*Comparison with the challenge group

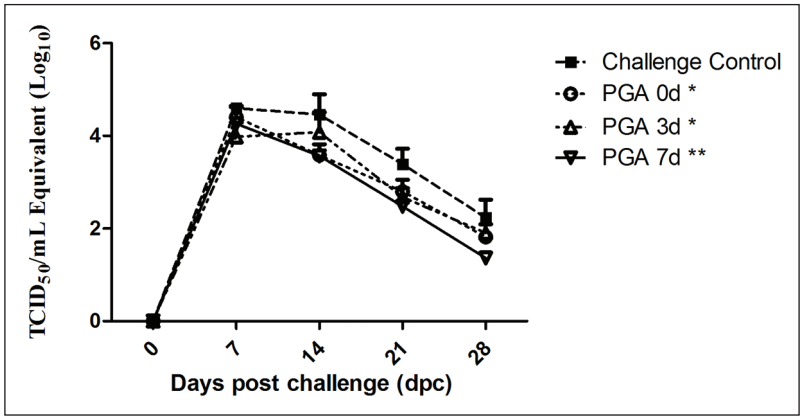

Fig. 2. The levels of viremia after PRRSV challenge (MN184) with and without PGA. The asterisks represent values significantly different (*: $p<0.05$, **: $p<0.001$) from that in the challenge control group.

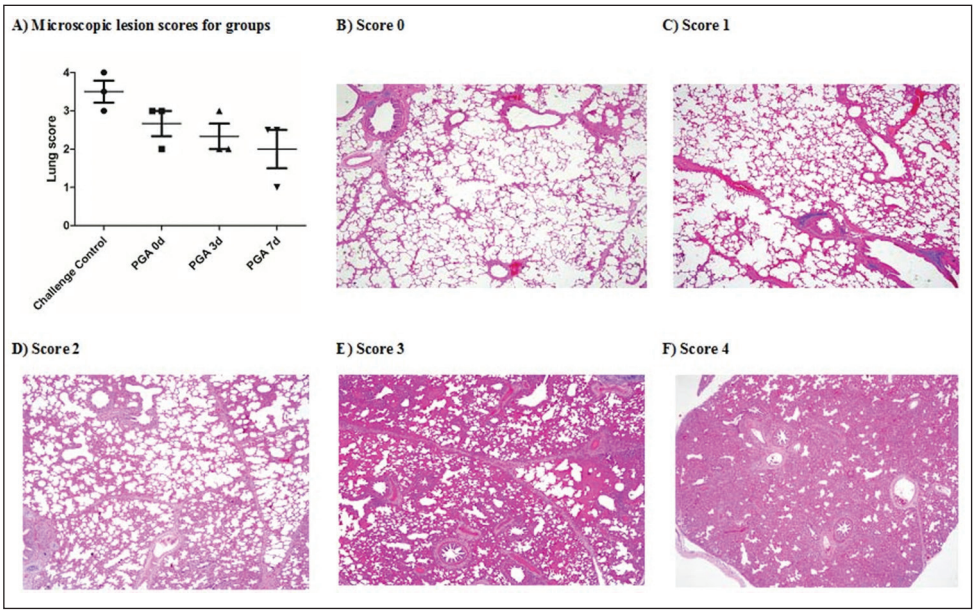

Fig. 3. Lung lesions after PRRSV challenge (MN184) with and without PGA treatment.

HM-γ-PGA enhanced SVN antibody induction in pigs infected with PRRSV

SVN antibody levels were determined in the sera collected 4 weeks after PRRSV infection. All groups treated with HM-γ-PGA exhibited higher levels of SVN antibody compared with the CC groups, and significantly higher levels of SVN antibody were observed in the group with HM-γ-PGA treatment 7 dpc (PGA 7d) than in the CC group ($p<0.05$) (Fig. 4).

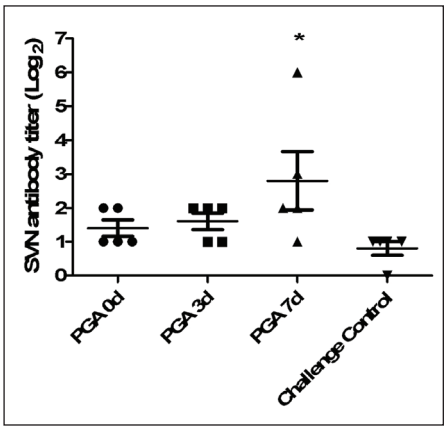

Fig. 4. The levels of SVN antibody 4 weeks after PRRSV challenge (MN184) with and without PGA treatment. The asterisk represents a value significantly different ($p<0.05$) from that in the challenge control group.

HM-γ-PGA enhanced cytokine responses in pigs infected with PRRSV

The mRNA expression levels of the pro-inflammatory cytokines IFN-α and TNF-α in PBMCs stimulated with MN184 were significantly higher in the groups treated with

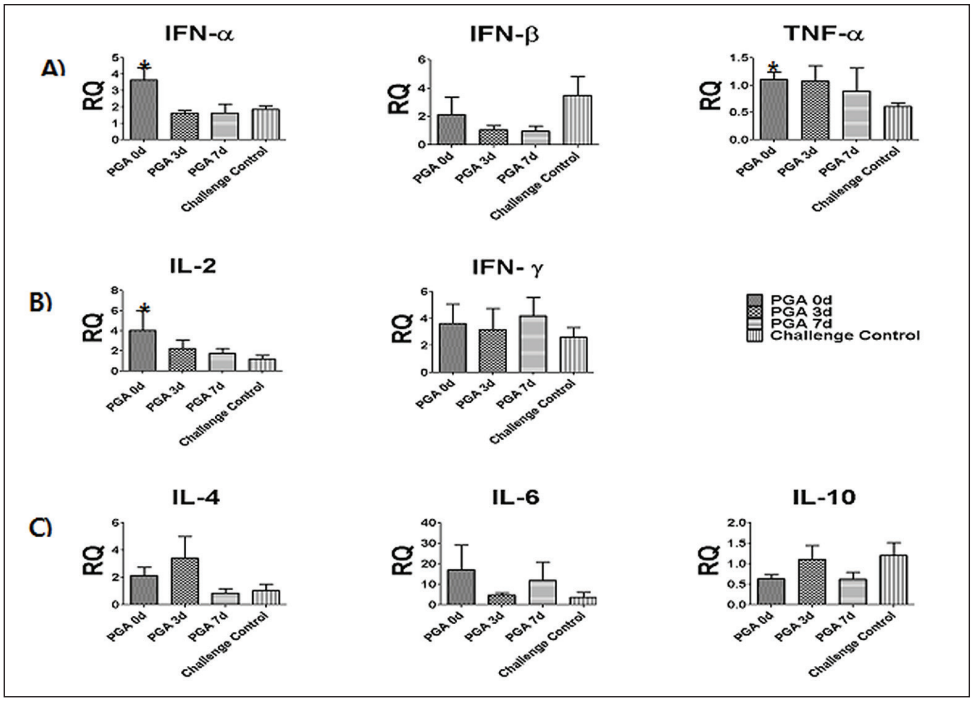

Fig. 5. Cytokine responses 3 weeks after PRRSV challenge (MN184) with and without PGA treatment. **A:** Proinflammatory cytokine responses; **B**: Type 1 cytokine responses; **C:** Type 2 cytokine responses. The asterisks represent values significantly different ($p<0.05$) from those in the challenge control group.

HM-γ-PGA at 0 dpc (PGA 0d) when compared with the CC group. The other groups treated with HM-γ-PGA at 3 or 7 dpc also displayed enhanced TNF-α expression levels when compared with the CC group. Among type-1 cytokines, the PGA 0d group displayed significantly higher interleukin (IL)-2 mRNA expression compared with the CC group, while all of the groups treated with HM-γ-PGA also showed generally higher IFN-γ mRNA expression than the CC group. Among type-2 cytokines, IL-4 and IL-6 mRNA expression levels were generally higher in the groups treated with HM-γ-PGA compared with the control challenge group, while mRNA expression of the regulatory cytokine IL-10 was higher in the CC groups compared with the groups treated with HM-γ-PGA (Fig. 5).

Biochemistry induction levels with HM-γ-PGA treatment

After challenge with MN-184, the CC groups showed higher levels of WBCs, RBCs, HGB, and HCT 21dpc when compared with HM-γ-PGA treatment groups, whose levels were similar. The levels of MCV and MCHC in serum were similar in all of the groups (Fig. 6).

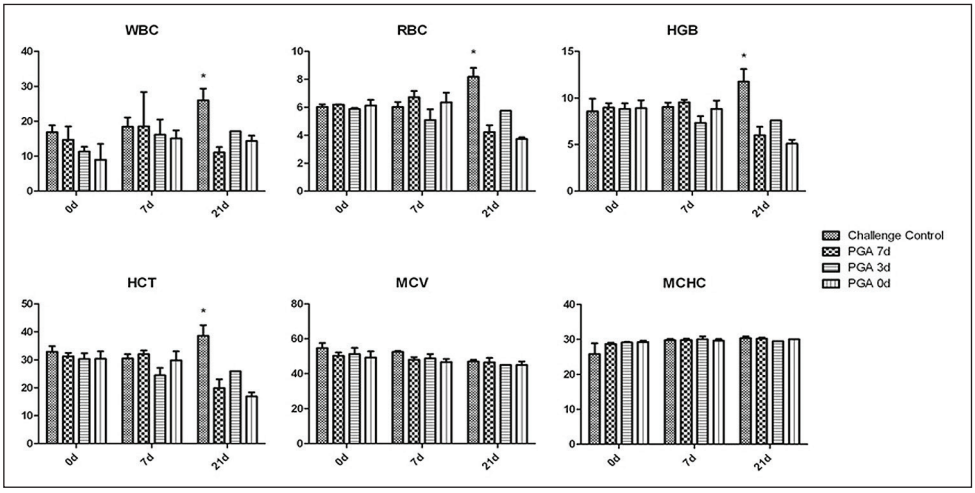

Fig. 6. Results of CBC test after PRRSV challenge (MN184) with and without PGA treatment. Asterisks represent values significantly different ($p<0.05$) among the groups.

DISCUSSION

In a previous study, the supplementation of 0.1% HM-γ-PGA had no effect on weight gain in rats compared to the 0% HM-γ-PGA groups [20]. However, our results clearly demonstrated that pigs injected with HM-γ-PGA gained more weight compared to the pigs in the NT group (Table 2). In addition, enhanced TNF-α, IFN-α and IFN-β mRNA expression levels were observed in pigs injected with 5 m*l* of HM-γ-PGA compared with groups injected with 1 or 3 ml of HM-γ-PGA (Fig. 1). Therefore, 5 ml of HM-γ-PGA was used in subsequent experiments.

The groups treated with HM-γ-PGA generally exhibited higher mRNA expression levels of pro-inflammatory cytokines (IFN-α, IFN-β, and TNF-α), type-1 cytokines (IL-2 and IFN-γ) and type-2 cytokines (IL-4 and IL-6) in PBMCs when compared with the CC group. Regulatory cytokine (IL-10) expression was higher in the CC groups than in the other groups treated with HM-γ-PGA (Fig. 4). These results may indicate that HM-γ-PGA injection increases overall immune responses in the pig. Consistent with this hypothesis, higher levels of neutralizing antibody were induced in the group treated with HM-γ-PGA at 7 dpi (PGA 7d) compared with the CC group (Fig. 3). Recently, HM-γ-PGA has been shown to stimulate antiviral activity by inducing type I IFN. In an HM-γ-PGA-treated mouse model, HM-γ-PGA-induced type I IFNs enhanced the antiviral state of the mice and protected them against a highly pathogenic influenza A virus [21]. Additionally, other studies have demonstrated that γ-PGA antiviral activity is mediated through the induction of innate immune responses via the TLR4-MD2 complex. Furthermore, γ-PGA with a molecular weight of 2000 kDa may be used as a broad-spectrum antiviral agent against viruses sensitive to type I IFNs [22]. Our results suggest that HM-γ-PGA exhibited potential antiviral effects against PRRSV based on *in vivo* studies. All groups treated with HM-γ-PGA had significantly higher weight gains, higher immune responses and increased resistance to PRRSV infection in pigs. Thus, these studies support potentially successful applications of HM-γ-PGA as a method of immune modulation against PRRSV infection.

Based on CBC test results, only pigs in the CC group exhibited significantly higher levels of WBCs, RBCs, HGB and HCT at 21 dpi, while the other groups treated with HM-γ-PGA had results within the normal range (Fig. 5). The higher levels of WBCs in the CC group may be evidence of systemic inflammation due to severe PRRSV infection. Higher levels of RBCs, HGB and HCT have often been observed in sick animals that are dehydrated due to a viral infection. However, the groups treated with HM-γ-PGA had test results within the normal range, indicating that HM-γ-PGA might reduce symptom severity and increase resistance to PRRSV infection in pigs.

In conclusion, the current study demonstrated the beneficial effects of HM-γ-PGA, a natural, edible, and biodegradable polymer derived from *Bacillus subtilis sups. chungkookjang* [1, 4, 23] in PRRSV infection. Pigs treated with HM-γ-PGA after PRRSV infection demonstrated more resistance when challenged with a highly virulent PRRSV strain, MN184, and exhibited higher weight gain and enhanced levels of protective immune responses.

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Authors' contributions

The manuscript reviewed and drafted by SBJ, LJH and KIJ. KWI supervised the work. Implementation of the study design and data recording was done by SBJ, LJH, KIJ, SN, KA and KIW. YMS, PC and KBS critically analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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EFEKTI POLI-γ-GLUTAMINSKE KISELINE VELIKE MOLEKULSKE MASE NA SVINJE SA REPRODUKTIVNIM I RESPIRATORNIM SINDROMOM (PRRSV)

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Bacillus subtilis sups. *chungkookjang* proizvodi poli- γ-glutaminsku kiselinu velike molekulske mase (γ-PGA). Prethodne studije su pokazale da postoji imunska stimulacija kao i antitumorski efekat γ-PGA što je dokazano eksperimentalno na miševima. Međutim, ovi nalazi nisu potvrđeni kod drugih vrsta životinja. Iz tog razloga, obavljena je studija sa ciljem da odredi uticaj γ-PGA na svinje koje su bile inficirane PRRS virusom kao i na zdrave svinje. PRRS negativne životinje su intramuskularno inokulisane sa 1, 3 ili 5 ml (20 mg/ml) γ-PGA. Jedna grupa svinja je služila kao negativna (NT) kontrola. Sve grupe koje su tretirane sa γ-PGA, imale su značajno veći prirast telesne mase, a svinje koje su tretirane sa 5 ml suspenzijom, imale su veće koncentracije faktora nekroze tumora (TNF)-alfa, α-interferona (IFN) kao i povećanu ekspresiju IFN-β, u poređenju sa ne tretiranom grupom (NT). Prema preliminarnim rezultatima, studija veštačke infekcije je obavljena upotrebom visoko virulentnih PRRS-virusnih sojeva (MN184), zajedno sa γ-PGA tretmanom u različitim periodima vremena tokom ogleda. Svinje koje su tretirane sa γ-PGA, imale su manje koncentracije virusa u serumu kao i u plućima uz značajno povećanje prirasta ($p<0.05$) u poređenju sa netretiranom grupom, a posle veštačke infekcije sa virulentnim virusom (MN184). Štaviše, svinje iz grupa koje su tretirane sa γ-PGA, posedovale su veće koncentracije neutralizacionih anti-PRRS virusnih antitela kao i citokine koji su povezani sa proinflamatornim, humoralnim i celularnim imunskim odgovorom, a u poređenju sa NT grupom, posle veštačke infekcije. Na osnovu rezultata, može da se zaključi da γ-PGA dovodi do stvaranja snažnijeg imunskog odgovora što znači da povećava rezistenciju na PRRS-virusnu infekciju svinja.