FULL PAPER *Immunology*

Effects of Sugar Cane Extract on the Modulation of Immunity in Pigs

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ABSTRACT. The experiment was aimed to test the efficacy of sugar cane extract (SCE) on the modulation of pig immunity under field conditions. The SCE preparation consisted of sugar cane extract (20%) and oilcake of rice bran (80%). SCE (500 mg/kg of body weight per day) was fed to weanling pigs on 3 consecutive days per week for 4 weeks. The results showed a significant enhancement of cytotoxicity of natural killer (NK) cells and phagocytosis by neutrophils and monocytes, compared to untreated pigs. The enhancement of NK cell function may have protected against porcine reproductive respiratory syndrome (PRRS), as there was a reduction in seroconversion rates in treated pigs. Moreover, SCE-treated pigs showed a 7.87% growth enhancement compared with untreated controls. Thus SCE produces an immunostimulative effect on porcine innate immunity that may provide protection against pathogens.
KEY WORDS: growth promotion, immunomodulation, leukocyte function, pig, sugar cane extract.

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Sugar cane extract (SCE) is a natural product which has displayed a wide range of biological effects including immunostimulation [8], anti-thrombosis activity [17], anti-inflammatory activity [14], vaccine adjuvant [7], anti-oxidant activity [18, 21], modulation of acetylcholine release [2], and anti-stress effects [3]. Study on mice inoculated with a minimum lethal dose of pseudorabies virus following with 3 consecutive days of oral administration with SCE showed a significant decrease in mortality (Koge, unpublished). Other studies have indicated SCE can enhance resistance to secondary bacterial infection [1, 19]. Studies on chickens indicate that SCE has an adjuvant effect on the activation of antibody and cell-mediated immune responses and provides a protective effect against *Eimeria tenella* infection [6]. These results suggest that SCE may positively regulate host natural immunity against viral, bacterial, and protozoal infections, via effects on the levels of macrophages, neutrophils and natural killer (NK) cells.

Porcine respiratory disease complex (PRDC) has become the major problem in the most intensive pig farms, causing massive economic loss. The causing factors of PRDC involve a complicated synergic interaction between host immunocompetence, polymicrobial infections and various stressors [23]. Pseudorabies, influenza virus, porcine reproductive respiratory syndrome (PRRS) virus, and *Mycoplasma hyopneumoniae* have been thought as primary agents of PRDC [10, 23]. However, antibiotics are commonly used to treat or prevent secondary bacterial infections of PRDC. As the abuse of antibiotics in veterinary medicine has been of particular concern from the viewpoint of public health, major strategies against infection are to improve the managerial systems and environmental conditions and to modulate host immunocompetence. Although studies of SCE on mice and chickens have shown enhancement of host defenses, the activity of SCE on the pig immune system and the efficacy of SCE applied in field conditions is still not documented. Therefore, the aim of this experiment was to investigate the effect of SCE on pig natural immunity, particularly on the function of neutrophils, monocytes, and NK cells. The efficacy of SCE application in farmed pigs was also evaluated.

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MATERIALS AND METHODS

Sugar cane extract (SCE): Shin Mitsui Sugar Co., Ltd., Japan has prepared four kinds of sugar cane extracts. Extract 1 from sugar cane juice consists of components adsorbed to a synthetic adsorbent resin. Extract 2 consists of volatile components from sugar cane juice adsorbed to a synthetic adsorbent resin. Extract 3 from sugar cane bagasse is obtained by hot water extraction. Extract 4 consists of crude protein (16.9%), fat (0.5%), ash (36.1%) and nitrogen-free extracts (46.5%) [8], prepared from sugar cane juice by chromatographic separation on an ion exchange column. Extract 4 was concentrated to ca. 40% solids containing ca. 4% sugars (glucose, fructose and sucrose). In this study, Extract 4 was used as SCE. SCE for oral administration was prepared by adsorption of Extract 4 to oilcake of rice bran, which was then dried. The ratio of SCE without glucose, fructose and sucrose: oilcake of rice bran was 1:4. Table 1 shows the composition of this material.

Experimental pigs, SCE administration, and sampling:

Table 1. Composition of sugar cane extract (Extract 4) for feed material

Data-item	% of sample	% of solid	Remarks
Moisture	6.7	–	135°C, 2 hr
Crude protein	18.5	19.8	Kjeldahl method
Crude fat	4.5	4.8	Diethyl ether extraction
Crude fiber (Insoluble fibers)	7.0	7.5	Filtration method
Ash	17.5	18.8	Direct incineration
Nitrogen-free extracts	45.8	49.1	*
Total	100.0	100	

*=100–(moisture + crude protein + crude fat + crude fiber +ash).

The experimental pigs for the kinetic study were purchased from a small farrow-to-finish pig farm that had been monitored for several years and had a history of good performance (>20 pigs/sow/year in the previous three years). Weanling pigs were raised under controlled conditions prior to and during the kinetic study. To test the effect of duration of SCE administration, pigs (N=5) were weighed and fed with SCE (500 mg/kg of body weight/day) for 1, 3, 5, or 7 consecutive days. To test the dose effect, 500, 1,000, 1,500 or 2,000 mg/kg of body weight/day was administered in feed for three consecutive days. Pigs fed with no SCE additive served as controls. Functional assays of leukocytes were conducted on day 4.

The effect of SCE on weanling pigs in the field was conducted on a farrow-to-finish pig farm of about 300 sows and 2,700 fattening pigs. The pig farm had a mild to moderate severity of PRDC. In this experiment, 180 weanling (five-week-old) pigs were randomly allocated into two groups, control (untreated, N=90) and SCE-treated (N=90), and moved into the isolated nursery unit. Ten pigs in each group were ear tagged for blood sampling. Pigs in the SCE-treated group were treated with SCE (500 mg/kg of body weight per day) in feed for three consecutive days per week for four weeks. The administration of SCE in feed was adjusted every two weeks, depending on the increasing body weight. Assays for leukocyte functions were performed at 1, 2 and 4 weeks post SCE administration. The growth performance, mortality, and the frequency of therapy in those pigs were recorded.

PRRS antibody detection: The presence of PRRS antibody in the control and SCE-treated pigs in the field test was assayed in 9-week-old pigs using a PRRS ELISA antibody kit (IDEXX Laboratories, Inc. U.S.A.), according to the manufacturer's instructions.

Leukocyte preparation: Blood samples were drawn from the jugular vein into heparinized tubes. Total white blood cell (WBC) was counted with an electronic haematology counter (Sysmex F-800). Heparinized blood was sedimented with 2% dextran in phosphate-buffered saline (PBS). After 15 min sedimentation, the upper layer of supernatant was separated by Ficoll-Paque (Pharmacia Biotech) gradient to obtain peripheral blood mononuclear cells (PBMCs) as previously described [11]. PBMCs were counted and the viability was determined by trypan blue exclusion method. PBMCs were suspended and adjusted to 1×10^7 cells/m*l* in RPMI 1640 medium (Gibco BRL, life Technologies, Inc.) containing 100 U/m*l* penicillin, 100 μg/m*l* streptomycin (Sigma, St. Louis, MO), and supplemented with 10% heat inactivated fetal bovine serum (Gibco BRL, Life Technologies, Inc.) and 2 mM L-glutamine (complete media; CM).

NK cells cytotoxicity: The assay was performed using a time-resolved fluorometer (TRF) system as previously described [11]. Briefly, the target cells, human erythromyelocytic leukemia cell line (K562) growing in logarithmic phase, were harvested and labeled with 2 μl of enhancing ligand bisacetoxymethyl 2,2': 6', 2"-terpyridine- 6,6"-dicarboxylate (BATDA) (Wallac Labelling Service) according to the manufacturer's instructions. Effector cells were obtained from PBMC after depletion of adherent cells in culture flasks for 30 min. The effector to target ratio was set at 100:1 and the mixture was incubated at 37°C in a 5% CO_2 incubator for 4 hr. Cytotoxic activity was calculated by the release of the fluorescent dye that had been chelated with europium (EuTDA) from dead target cells. The percent specific release of fluorescence of EuTDA was measured in a time-resolved fluorometer (1234 DELFIA, Wallac, Turku, Finland) and calculated as: [(Experimental release - spontaneous release)/(maximum release - spontaneous release)] × 100%. Throughout the experiment, counts of spontaneous release were ranged between 18% and 25%.

Phagocytosis by monocytes and neutrophils: Phagocytosis by monocytes and neutrophils was performed in whole blood and assayed by flow cytometry. A stock of FITC (fluorescein isothiocyanate) -labelled *Salmonella choleraesuis* (SC; ATCC 10743) stored in 20% glycerol (4×10^9 CFU/m*l*) was resusciated and opsonized with antiserum containing polyclonal antibody to *Salmonella*. One hundred microliters of blood was incubated with 25 μl of SC in a microtube (Bibby Sterilin Ltd, England; 50 × 6 mm round base) (leukocyte:bacterium ratio=1:25) at 37°C for 30 min. After incubation, RBCs were lysed in a buffer containing 0.899% ammonium chloride, 0.1% potassium hydrogen carbon and 0.0037% disodium EDTA in distilled water (DW) then washed in chilled FACS washing buffer (PBS containing 0.1% (w/v) bovine serum albumin and 0.01% (w/v) sodium azide). Cells were fixed in 1% paraformaldehyde in PBS and stained with monoclonal antibody (74–22–15;

ATCC) to SWC3, a surface molecule of phagocytes, followed by goat anti-mouse IgG-phycoerythrin (PE) conjugate, $F(ab')_2$ (1:100; Sigma Chemical Co.). Ten thousand PE-positive cells were collected by flow cytometry (Becton Dickinson Immunocytometry system; BDIS) using a CellQuest software (BDIS). Cell populations enriched for neutrophils and monocytes were gated on a side scatter (SSC) and SWC3 (FL-2) scatter plot. The background control was set on phagocytes which had not been fed bacterium. The percentage of FITC-positive cells (FL1) and mean fluorescence were measured against a 1% positive cell gated on background control. The phagocytic activities of cells were expressed as a phagocytic index (PI) calculated as: [(% positive × mean channel fluorescence) / 100].

Statistical analysis: Statistical analyses of the data were calculated using analysis of variance (ANOVA) and Duncan's multiple-range tests with P value of <0.05 being used to determine significance.

RESULTS

Kinetic effects of SCE administration on leukocyte function: As data on the effect of SCE in pigs is lacking, the kinetic effect of SCE dose (500, 1,000, 1,500, and 2,000 mg/kg/day) and duration (1, 3, 5, and 7 consecutive days) of SCE administration were evaluated in pigs in a controlled environment. The results showed that both the cytotoxic activity of NK cells and phagocytosis by monocytes were significantly increased in pigs treated with SCE for 1, 3, 5, and 7 days, compared with untreated control pigs ($p<0.05$). The duration of SCE administration did not produce any significant differences (Fig. 1A, 1B). Enhancement of phagocytic activity was not noted in neutrophils ($p>0.05$) (Fig. 1C).

To further understand the effect of dose of SCE, a preliminary study was conducted on pigs in a controlled environment. Five-week-old pigs were fed with different dosages of SCE (500 mg, 1,000 mg, 1,500 mg, or 2,000 mg/kg of body weight per day) for 3 consecutive days. Leukocyte function was assayed on day 4. The results showed a significant enhancement of NK cell cytotoxicity in those pigs fed with 500 or 1,000 mg SCE/kg/day compared with controls ($p<0.05$). However, the enhancement of NK cell function was not seen in pigs fed with high dosages of SCE (1,500 mg/kg and 2,000 mg/kg) (Fig. 2A). Phagocytic function of monocytes was significantly enhanced in pigs fed with SCE at a dose of 1,000 mg/kg ($p<0.05$), but not in pigs fed with SCE at doses of 500, 1,500 or 2,000 mg/kg/day ($p>0.05$) (Fig. 2B). Meanwhile, the administration of different doses of SCE (500 mg/kg, 1,000 mg/kg, or 1,500 mg/kg) did not cause any changes in phagocytosis by neutrophils. Controversially, there was a significant decrease ($p<0.05$) in phagocytic function of neutrophils in pigs fed with high dose of SCE (2,000 mg/kg), relative to control pigs (Fig. 2C).

Efficacy of SCE administration on immunomodulation under field conditions: Based on the results of kinetic study at a commercial pig farm, weanling pigs were fed with 500 mg/kg/day of SCE, and effects on innate immunity were evaluated. The cytotoxicity of NK cells in pigs fed with SCE for 1, 2, and 4 weeks was significantly enhanced compared to control groups ($p<0.05$) (Fig. 3A).

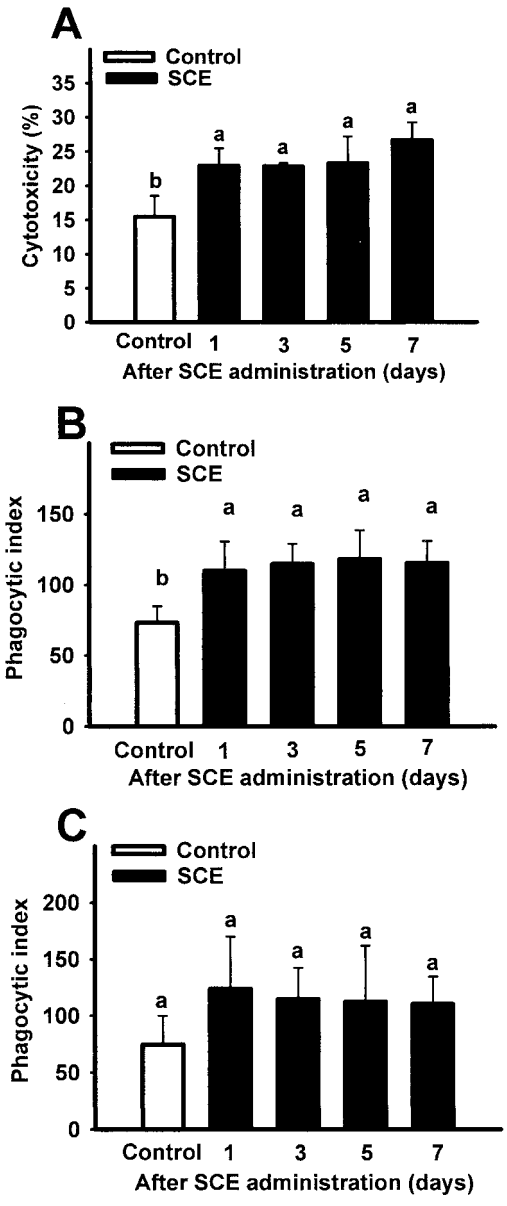

Fig. 1. Effect of duration of SCE administration on leukocyte activity. Five pigs in each group were fed with SCE (500 mg/kg/body weight/day) for 1, 3, 5, and 7 days. Leukocyte activity assays, including cytotoxicity of NK cells (A), phagocytosis by monocytes (B) and neutrophils (C), were performed at the same days. Different superscripted letters indicate a significant differences ($p<0.05$) between the control group (open bar) and the SCE-treated group (solid bar).

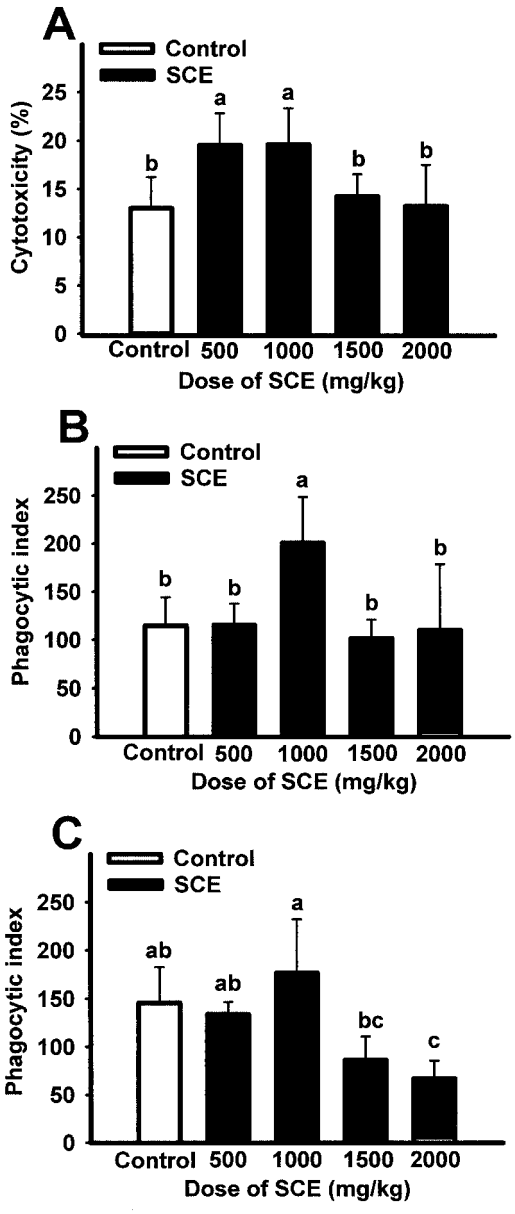

Fig. 2. Effect of SCE dose on leukocyte activity. Five pigs in each group were fed with different dosages of SCE (0–2,000 mg/kg/body weight/day) for 3 consecutive days. Leukocyte activity assays, including cytotoxicity of NK cells (A), phagocytosis of monocytes (B) and neutrophils (C), were performed at day 4. Different superscripted letters indicate a significant differences ($p<0.05$) between the control group (open bar) and the SCE-treated group (solid bar).

The phagocytic activity of monocytes from pigs fed with SCE for 2 and 4 weeks showed a 58.24% and 49.55% increase, respectively, which was significantly different from controls ($p<0.05$) (Fig. 3B).

Analyses of phagocytic activity of neutrophils, showed an increase in pigs fed with SCE for 2 weeks (76.46% increase) and 4 weeks (49.32% increase), similar to the trend seen in monocytes (Fig. 3C).

Anti-viral effect of SCE under field conditions: To address whether SCE can lead to a restriction of viral replication by enhancing NK cell activity, the effect of SCE on infection by PRRS virus was evaluated. As the trial farm had been contaminated with PRRS virus, piglets would be exposed to the virus and develop antibody later. In this experiment pigs had not received PRRS vaccine. Weanling pigs were fed with SCE (500 mg/kg/day) for 3 consecutive days per week for 4 weeks, and blood samples were collected for PRRS antibody detection. Pigs fed with SCE showed 8.33% seroconversion, which is much lower than the 25% seroconversion rate observed in control pigs (Fig. 4). However, the prevalence of PRRS antibodies did not differ between the treated and control groups.

Effect of SCE on growth and health: Growth performance including morbidity, mortality and body weight was evaluated between 5 and 14 week-old pigs. Two pigs in each group were culled due to diarrhoea, dermatitis or respiratory distress. There was no obvious difference in morbidity and mortality between the SCE-treated and the control groups. However, the average body weight of SCE-treated pigs increased by 7.87% compared with the control group, but this difference was not statistically significant ($p>0.05$) (Fig. 5).

DISCUSSION

Some plant extracts and probiotics have been found to have a wide range of physiological functions. Those products have been widely used as supplements in animal foods to boost innate immunity against infections [9, 15, 24]. By-products of sugar production from sugar cane have been reported to have a wide range of biological activities [12, 14, 17, 22], especially antioxidative activities, phylactic activities, and other physiological functions [1, 18, 19, 21]. Protective phylactic effects against viral and bacterial infections could be exploited to reduce the use of antibiotics in the pig industry. The results reported here, showing that SCE induces an up-regulation of leukocyte functions, highlights the potential benefits of SCE to the pig industry.

In the chicken model, a dose of SCE between 500 and 1,500 mg/kg of body weight produces adjuvant and phylactic effects against viral, bacterial, and protozoal infections [6–8]. Decreasing the dose of SCE may reduce the protective effect against infections. The administration of SCE in pigs has not been documented previously. Our results demonstrated a significant effect on NK cell cytotoxicity and the phagocytic activity of monocytes at 500 and 1,000 mg/kg/body weight of SCE. However, in contrast to studies on chickens, no significant enhancement of phagocytic activity of neutrophils was observed in pigs ($p>0.05$) [8]. The lack of effect of SCE on neutrophil activity in this study may be due to sample sizes, species differences between pigs and chickens, and high variation in phagocytic activity of neu-

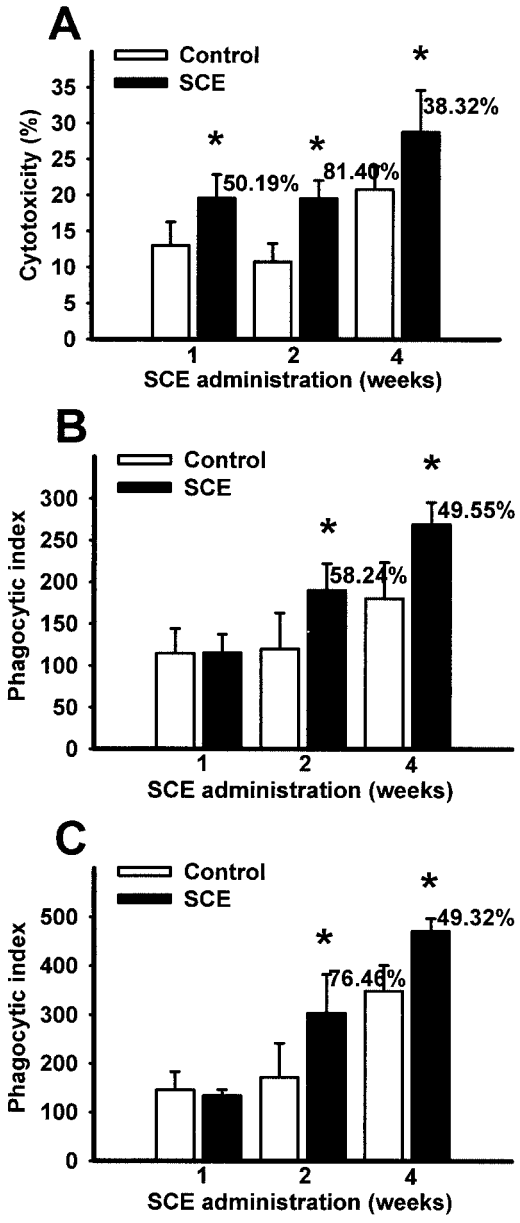

Fig. 3. Effect of prolonged treatment with SCE on leukocyte function. Weanling pigs were fed with SCE (500 mg/kg/ body weight/day) for 3 consecutive days per week for 4 weeks. Pigs fed without SCE served as controls. Leukocyte activity assays, including cytotoxicity of NK cells (A), phagocytosis of monocytes (B) and neutrophils (C), were performed at week 1, 2 and 4. Asterisk (*) indicates a significant difference ($p<0.05$) between the untreated control group (open bar) and the SCE-treated group (solid bar).

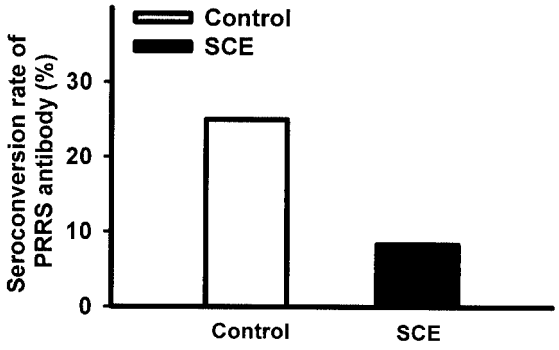

Fig. 4. Effect of SCE on PRRS virus infection. Weanling pigs were fed with SCE (500 mg/kg/day) for 3 consecutive days per week between the ages of 5 and 9 weeks. Blood samples were collected at the end of week 9. The presence of PRRS antibody was assayed using an ELISA kit.

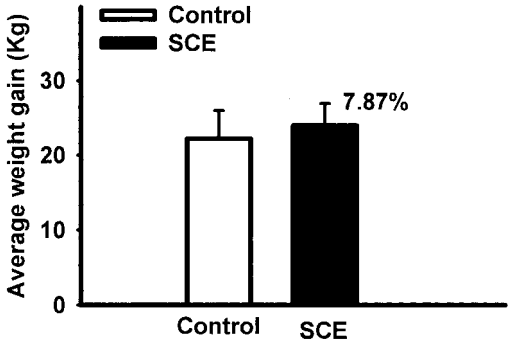

Fig. 5. Evaluation of SCE-treatment on growth performance of pigs. SCE was administered for 3 consecutive days per week between the ages of 5 and 9 weeks, and the body weight was measured in 14-week-old pigs. There was a 7.87% increase in growth in the SCE-treated group compared to control group, but this was not statistically significant ($p>0.05$).

trophils in pigs [5]. Moreover, between pigs and chickens, phagocytosis by porcine neutrophils may be positively regulated with a longer treatment, as noted under field conditions. The enhancement of leukocyte function in pigs fed with SCE at dosages of 500 and 1,000 mg/kg body weight, but not with 1,500 and 2,000 mg/kg body weight, suggests that some functions may decrease with high dosages. The reason behind this is not clear, however similar phenomena have been observed in other studies. High dosages of β-glucan caused overstimulation of prostaglandin E production and down-regulation of β-glucan receptor expression, resulting in the inhibition of phagocytosis [13]. High doses of another immunostimulant from *Ganodema lucidum* caused a decrease in leukocyte function [15]. Moreover, if SCE contains high levels of mannan, high dosages of the preparation may competitively bind the mannan receptor leading to down regulation of phagocytosis by macrophages and the production of IL-12, TNF-α and IFN-γ by lymphocytes [20]. Therefore, an appropriate dose and duration of SCE administration may be important for the efficient enhancement of porcine leukocyte function.

As weanling pigs face the gradual decay of maternal antibodies, there is increased risk of viral infection, particularly PRRS virus or porcine *circovirus* infection, which may persist in nursery units. The pilot studies showed that SCE has an immunostimulative effect at particular dosages, and under field conditions the mortality and morbidity rates in both control and SCE-treated groups were low, possibly due in part to depopulation and sanitation of the nursery before this experiment. It seems logical that good managerial and sanitary procedures are among basic requirements for disease control. Although leukocyte functional assays also confirmed that SCE could significantly enhance innate immunity including NK cells, monocyte and neutrophil functions at 2 weeks post SCE administration, phagocytosis by neutrophils and monocytes had not increased by 1-week post SCE administration. Variation among individuals, the presence of stressors, and managerial factors in field conditions may have influenced this result. Moreover, the cytotoxicity of NK cells of pigs at 4 weeks post experiment in both SCE treated and untreated control pigs was greatly enhanced compared with cytotoxicity at 1 and 2 weeks post-treatment. The increase in cytotoxicity may relate to increasing age as has been previously reported [11]. The significant enhancement of innate immunity after SCE stimulation may relate to cytokine release from activated leukocytes, in turn amplifying leukocyte functions [13, 16, 20].

PRRS has been thought of as an important primary pathogen in PRDC [23]. The serological and pathological data show a high prevalence of PRRS antibodies in most pig farms in Taiwan [4]. This may reflect exposure to PRRS virus under nursery conditions. As levels of cytotoxicity of NK cells are consistently increased after administration of SCE, the anti-viral activity of SCE towards PRRS is of particular interest. Both groups of experimental pigs were raised in the same pig house and exposed to the same air conditions. The seroconversion rate was greatly decreased in SCE-treated pigs. A repeated experiment at another pig farm also showed similar results (data not shown), confirming the antiviral effect of SCE in pigs. This suggests that the administration of SCE can enhance NK cell activity against early PRRS virus infection, and may contribute to decreasing the incidence of PRDC in pig farms.

Pigs may be gradually infected with PRRS virus and other pathogens at the nursery stage and develop antibodies later, as discussed above. Therefore, to evaluate the influence of natural virus infection, the effect of SCE on growth performance was evaluated at the end of the experiment, being week 14. In the farmed pigs, SCE-treated animals displayed 7.87% enhancement of growth compared to control pigs, but this was not a statistically significant difference. Molasses has been widely used in feed to improve pig appetite and SCE also shows a similar taste improvement that may contribute to increasing pig growth rate. Additionally, the reduction of infections and severity of pulmonary lesions after SCE administration may have further contributed to improving growth. A similar improvement of growth has been reported in chickens fed SCE through the positive regulation of host natural immunity against bacterial and protozoal infections [6, 8].

In conclusion, SCE has a broad biological effect in raising innate immunity to infections. Besides the improvement of managerial and environmental systems, and vaccination against infections, the administration of immunostimulants in feed may be an alternative method of preventing and reducing infections in pigs.

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