

Immunostimulatory effects of an anionic alkali mineral complex solution (Barodon[®]) on porcine lymphocytes

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Summary

The anionic alkali mineral complex solution, Barodon[®], increased expression of porcine leukocyte subpopulations in treated pigs, determined by use of specific monoclonal antibodies and flow cytometry. This immunostimulatory effect supports use of Barodon as an alternative to antimicrobials for improving productivity in the swine industry.

Keywords: swine, Barodon[®], immunostimulator, CD4⁺CD8⁺ double-positive population

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There has been an increasing demand in the food animal industry for drugs which leave no residue in meat, because of concern about antibiotic-resistance problems in humans.¹ Alternatives to antimicrobials, such as nonspecific immunostimulators, synthetic peptides, natural herbs, and fermentative microorganisms, are being evaluated with new interest.²⁻⁹ Dietary supplements, such as probiotics, vitamins, amino acids, and minerals, are relatively well established in the feed industry, show considerable potential for improving growth rate of animals, and may enhance immunity, alter micro-

bial flora, and reduce the occurrence of diseases associated with pathogens.⁹⁻¹³

Recently, an anionic alkali mineral complex solution, Barodon (Barodon-SF, Ansung, Gyeonggi, Korea), was introduced to improve productivity of food-producing animals in Korea. Barodon's properties are based on its mineral composition, including silicon, silver, sodium, and potassium ions in an alkaline solution (pH 13.5). Although Barodon was patented as an anionic solution in the US,¹⁴ and also in Korea,¹⁵ the exact mechanism of its effects are unknown. This study was designed to evaluate Barodon as a nonspecific immunostimulating agent in pigs. A set of monoclonal antibodies specifically reactive with porcine leukocyte differentiation antigens were used with flow cytometry to determine the proportions of leukocyte subpopulations. To investigate the specific cell types which may respond to Barodon, two-color fluorescence flow cytometry was used with monoclonal antibodies (mAbs) of different isotypes that reacted with lymphocytes from peripheral blood and lymphoid tissues from treated feeder pigs.

Materials and methods

Animals and housing

A total of 50 mixed-breed (Yorkshire-Landrace-Duroc), healthy feeder pigs at 15

weeks of age were used for the study. Pigs were randomly divided into a Barodon-treated group (40 pigs) and a control group (10 pigs). Pigs were housed ten per pen in five 4 × 4.2-m pens (16.8 m²) with concrete floors in an environmentally-controlled building.

Feeding and treatment with Barodon

The control group was fed a grower diet (Leantec Grower; Agribands Purina Korea, Seoul, Korea) based on corn and soybean oil meal, with 17% crude protein, 6% fat, 3% fiber, and digestible energy 3500 kcal per kg. Minerals (including calcium, phosphorus, iron, magnesium, copper, and zinc) and vitamins (including vitamins A, D₃, and E) were added at levels commonly found in grower diets. The Barodon-treated group was fed the same diet sprayed with Barodon at a rate of 0.05% of the total amount of feed. Composition of Barodon is shown in Table 1. Barodon contains sucrose, which was present in the treated feed at 15.97 g per 1000 kg of feed, for a difference in energy of 0.16 kcal per day per pig in the Barodon-treated group.

Experimental design

The pigs were allowed 1 week of acclimatization in the housing facility, and had free access to feed and water. During Study Weeks 1 to 9 of the 13-week study, the four treated pens received the grower diet with 0.05% Barodon, and the control pen received the same diet without Barodon. During Study Weeks 10 through 13, both Barodon-treated and control groups received untreated feed. Daily weight gain and feed consumption were measured and feed conversion rate was calculated on an individual animal basis in each group until the end of Week 5. Blood samples were collected at the beginning of the study and at Study Weeks 3, 8, 11, and 13. During each of Study Weeks 8 and 11, two pigs from the control group and four from the

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SIC: Barodon-SF Corp, Ansung, Gyeonggi, 456-880, Korea.

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Table 1: Major ingredients of Barodon,¹ a complex anionic alkali mineral aqueous solution² used as a non-specific immunostimulator in pigs

Ingredient	Concentration (g/L of water)
Sodium metasilicate (Na ₂ SiO ₃)	600
Potassium carbonate (K ₂ CO ₃)	300
Sodium carbonate (Na ₂ CO ₃)	9
Sodium borate (Na ₂ B ₄ O ₇)	9
Sucrose (C ₁₂ H ₂₂ O ₁₁)	900
Silver nitrate (AgNO ₃)	0.01
Sodium chloride (NaCl)	0.03
Sodium thiosulphate (Na ₂ S ₂ O ₃)	0.12

¹ Barodon-SF Corp, Ansong, Gyounggi, 456–880, Korea.

² Specific gravity 1.43, pH 13.5.

Barodon-treated group were sacrificed for collection of mesenteric lymph nodes (a total of 12 pigs). The remaining pigs in both groups were weighed at the end of the study period (Week 13).

Preparation of peripheral blood leukocytes and mesenteric lymph node leukocytes

Approximately 20 mL of blood was collected from each animal using Vacutainers (Becton Dickinson Vacutainer System, Rutherford, New Jersey). Peripheral blood leukocytes were separated by the method of Davis et al (1987).¹⁶ Briefly, blood was mixed with an equal volume of acid-citrate-dextrose-EDTA and leukocytes were separated using density gradient centrifugation in Hypaque Ficoll (specific gravity 1.086; Sigma Chemical, St Louis, Mis-

souri) at 800g for 30 minutes. Mesenteric lymph node leukocytes were also separated by the method of Davis et al¹⁶ using Hypaque Ficoll. Cells were counted by the trypan blue exclusion technique¹⁶ and final concentration was adjusted to 1×10⁷ cells per mL.

Monoclonal antibodies specific to porcine leukocyte differentiation antigens

A panel of mAbs (Veterinary Medical Research and Development, Inc, Pullman, Washington) specifically reactive with porcine leukocyte differentiation antigens is shown in Table 2. The mAbs specific to major histocompatibility complex (MHC)-class I, MHC-class II, porcine (Po)CD2, PoCD4, PoCD8, surface (s)IgM, nonT/nonB (γδT-cell receptor), granulocyte, and

monocyte antigens were used to examine the proportions of leukocyte subpopulations in peripheral blood or mesenteric lymph nodes. Mouse anti-pig CD4-fluorescein isothio-cyanate (FITC) conjugate (isotype IgG2a; Southern Biotechnology Associates, Birmingham, Alabama) and CD8-phycoerythrin (PE) conjugate (isotype IgG2b; Southern Biotechnology Associates) were used in dual color analysis.

Flow cytometry analysis

The proportions of leukocyte subpopulations were determined by flow cytometry using the CellQuest program (FACS Calibur, Becton Dickinson Immunocytometry System, San Jose, California). Approximately 50 μL of each mAb (15 μg per mL) was reacted with 100 μL of cells (1×10⁷ cells per mL) in a V-bottomed, 96-well microplate (Costar, Corning, Corning, New York). Plates were first incubated on ice for 30 minutes, then washed three times with PBS-ACD buffer with horse serum (PBS, 450 mL; acid-citrate-dextrose (ACD), 50 mL; 20% sodium azide, 5 mL; gamma globulin-free horse serum [Sigma], 10 mL; 250 mM EDTA, 20 mL; and 0.5% phenol red, 1 mL), with centrifugation at 700g for 5 minutes. Pellets were disrupted by vortexing and mixed with 100 μL of FITC-conjugated goat anti-mouse IgG+IgM antibody (Caltag Laboratories, Burlingame, California) that had been diluted 1:200 in buffer and incubated on ice for 30 minutes in the dark. Pellets were then washed three times with PBS-ACD buffer without horse serum with centrifu-

Table 2: Monoclonal antibodies specifically reactive with swine leukocyte differentiation antigens

MAb ¹	Isotype of mAb	Molecules ²	Cell type ³
PT85A	IgG _{2a}	MHC class I	All nucleated cells
H42A	IgG _{2a}	MHC class II	Antigen-presenting cells
TH81A5	IgG _{2a}	MHC class II	Antigen-presenting cells
MSA4	IgG _{2a}	PoCD2	T cells
PT90A	IgG _{2a}	PoCD4	Th/i cells
PT81B	IgG _{2b}	PoCD8	Tc/s cells
Plg45A	IgG _{2b}	slgM	B cells
PT79A	IgG _{2a}	γδ TCR	γδ cells
DH59B	IgG ₁	Granulocyte + monocyte ⁴	Granulocyte + monocyte

¹ Veterinary Medical Research and Development Inc, Pullman, Washington.

² Porcine leukocyte differentiation molecules: MHC: major histocompatibility; PoCD: porcine cell differentiation; slgM: surface IgM; TCR: T-cell receptor.

³ Types of cells expressing molecules.

⁴ Molecules specifically found on the surface of granulocytes and monocytes.

gation at 700g for 5 minutes. After the final washing, pellets were mixed with 200 μ L of 2% PBS-formaldehyde (37% formaldehyde, 20 mL; PBS, 980 mL) and stored at 4°C for flow cytometry analysis.

For dual color analysis, a pair of FITC- or PE-conjugated PoCD4 or PoCD8 mAbs of different isotypes were used as second step reagents.

Statistical analysis

The proportions of lymphocytes carrying the various antigens in peripheral blood and the proportions of CD4⁺CD8⁺ double positive T lymphocytes in peripheral blood and mesenteric lymph nodes collected during the 13 weeks of the study were compared in pigs in the Barodon-treated and control groups using the Kruskal-Wallis one-way ANOVA by ranks. Analyses were performed using Microcal Origin 5.0 (Microcal Software, Northampton, Maine), with the level of significance set at $P < .05$.

Results

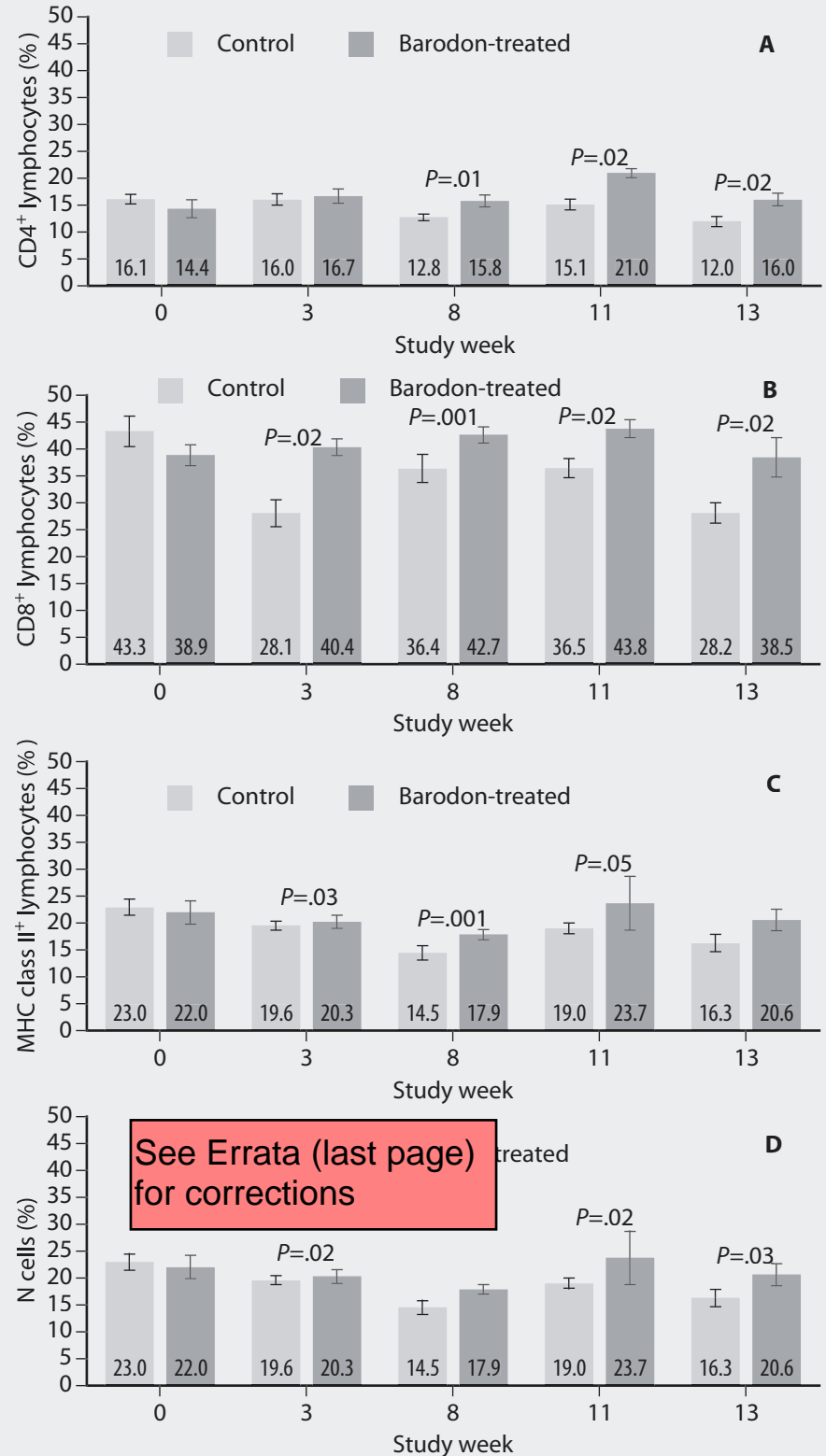
Feed efficiency, weight gain, and productivity

Pigs remained clinically healthy during the study. During the first 5 study weeks, Barodon-treated pigs gained 890.48 g per day (SD 41.92 g), and control pigs gained 842.86 g per day (SD 61.42 g); feed intake was 2769.05 g per day for Barodon-treated pigs and 2713 g per day for control pigs; and feed-to-gain was 3.11 for the Barodon-treated pigs and 3.22 for control pigs. At the end of the 13-week study, mean weight was 108.75 kg (SD 5.13 kg) for the remaining 32 Barodon-treated pigs and 106.20 kg (SD 8.38 kg) for the remaining six control pigs.

Flow cytometry analysis

The proportion of peripheral blood lymphocytes expressing CD4 antigen was higher in the Barodon-treated group than in the control group at Study Weeks 8, 11, and 13 (Figure 1A). The proportions of peripheral blood lymphocytes expressing CD8 antigen were higher in the Barodon-treated group than in the control group at Study Weeks 3, 8, 11, and 13 (Figure 1B). The proportions of peripheral blood lymphocytes expressing MHC class II antigen were higher in the Barodon-treated group than in the control group at Study Weeks 3, 8, 11, and 13 (Figure 1C). In addition, the proportions of non T/non B cells were

Figure 1: Proportions of (A) CD4⁺, (B) CD8⁺, and (C) MHC-Class II⁺ lymphocytes, and (D) nonT/nonB (N) cells in peripheral blood of pigs treated with Barodon (40 pigs) and in untreated controls (10 pigs). The same grower diet was fed to each group, with Barodon sprayed on the feed at a rate of 0.05% for the treated group for the first 9 weeks of the study. During Study Weeks 10 to 13, both groups received the untreated diet. Pigs were approximately 15 weeks old at the beginning of the study. Composition of Barodon (Barodon-SF, Ansung, Gyeonggi, Korea) is shown in Table 1.



higher in the Barodon-treated group than in the control group at Study Weeks 3, 11, and 13, but not at Study Week 8 (Figure 1D). There were no differences between the Barodon-treated group and the control group in the proportions of peripheral blood CD2⁺ cells, B cells, monocytes, or granulocytes during the experiment (data not shown).

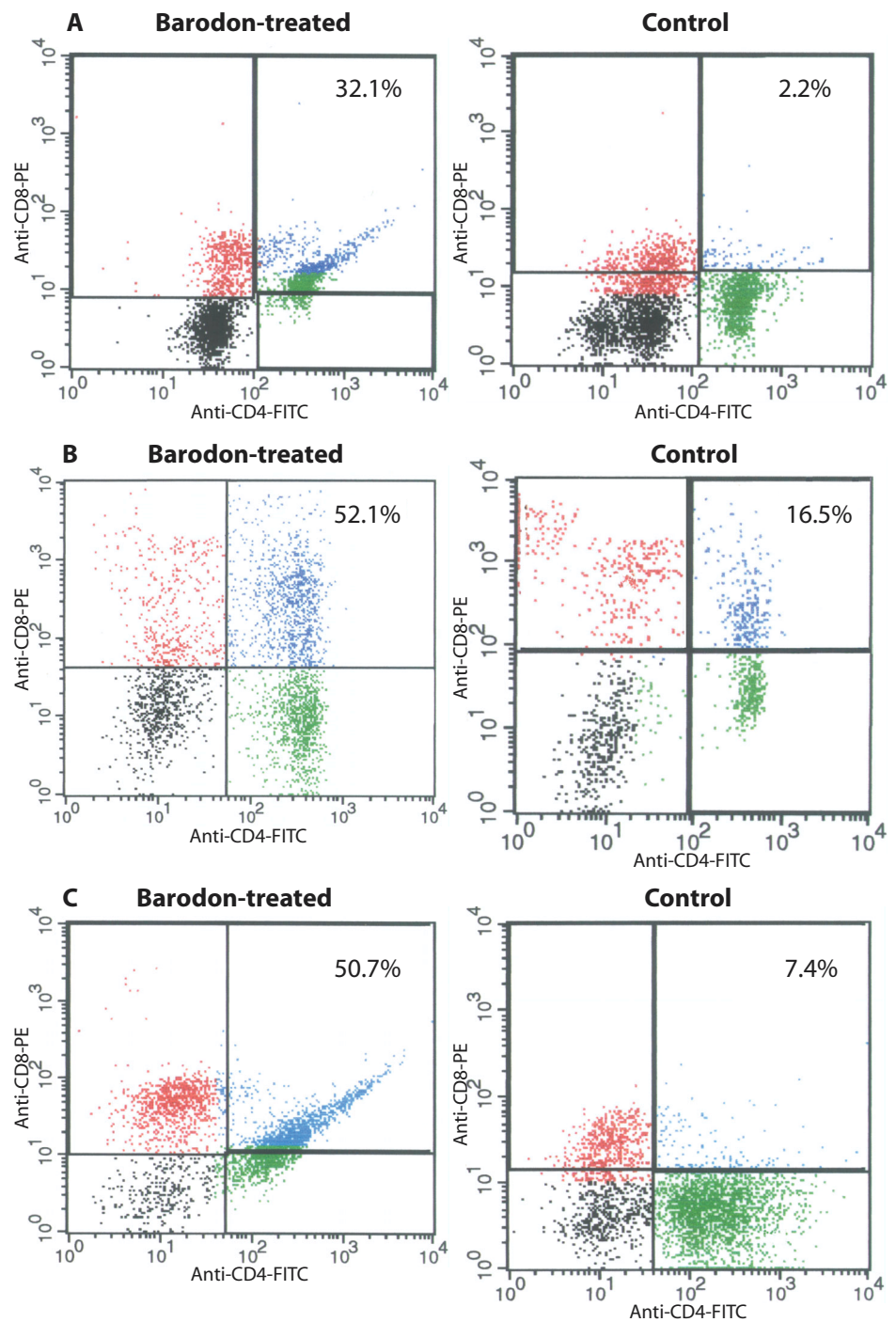
Proportion of CD4⁺CD8⁺ double-positive T lymphocytes in peripheral blood and mesenteric lymph nodes

At Study Week 8, a higher proportion ($P < .05$) of CD4⁺CD8⁺ double-positive T lymphocytes was observed in the Barodon-treated group than in the control group, both in peripheral blood lymphocytes (Figure 2A) and in mesenteric lymph node lymphocytes (Figure 2B). At Study Week 11, a higher proportion ($P < .05$) of double-positive lymphocytes was observed in mesenteric lymph node lymphocytes in the Barodon-treated group than in the control group (Figure 2C).

Discussion

The recent development of mAbs specific to leukocyte differentiation antigens of various animals, including pigs, makes it possible to define the host immune system more completely.¹⁷⁻²⁷ By monitoring the animal immune system, the efficacy of vaccines and new drugs can be evaluated in vivo by comparing the host response before and after application of reagents.^{20,22,28} The porcine immune system is unique in the expression of extrathymic CD4⁺CD8⁺ double-positive lymphocytes.^{21,27,29-31} This double-positive population is found in the peripheral blood and secondary lymphoid organs of swine,^{21,30,31} and the proportion increases with age.^{21,30-33} In 1-month-old piglets, 1% of T cells are CD4⁺CD8⁺, while in 3-year-old pigs, up to 60% of T lymphocytes express these antigens.²¹ The age-associated increase in the proportion of CD4⁺CD8⁺ cells suggests that this type of T cell may have an important role in host defense. In fact, the CD4⁺CD8⁺ population does play a major role in protective immunity.³⁰ Several studies have showed that these cells possess immunological memory function.^{21,31} Zuckermann and Husmann³¹ showed that CD4⁺CD8⁺ T lymphocytes have a specific memory cell marker, CD29; thus, the double-positive population may play a role

Figure 2: Dot plot profiles representing proportions of CD4⁺CD8⁺ double positive populations in (A) peripheral blood lymphocytes at Study Week 8, (B) mesenteric lymph nodes (MLN) at Study Week 8, and (C) MLN at Study Week 11 in groups of pigs treated with Barodon or untreated. Pigs were approximately 15 weeks old at the beginning of the study. The same grower diet was fed to each group, with Barodon sprayed on the feed at a rate of 0.05% for the treated group for the first 9 weeks of the study. During Study Weeks 10 to 13, both groups received the untreated diet. Composition of Barodon (Barodon-SF, Ansong, Gyeonggi, Korea) is shown in Table 1. Dual color analysis was performed with mouse anti-pig CD4-fluorescein isothiocyanate (FITC) conjugate (isotype IgG2a) that reacted with CD4⁺ cells, and mouse anti-pig CD8-phycoerythrin (PE) conjugate (isotype IgG2b) that reacted with CD8⁺ cells. Blue dots represent CD4⁺CD8⁺ lymphocytes, green dots represent CD4⁺CD8⁻ lymphocytes, red dots represent CD4⁻CD8⁺ lymphocytes, and black dots represent CD4⁻CD8⁻ lymphocytes. The numbers in the upper right quadrants indicate the proportions of CD4⁺CD8⁺ double positive cells.



in inducing secondary immune responses in the host.

Although the total CD2⁺ T lymphocyte population was not larger in the Barodon-treated group, the CD4⁺ and CD8⁺ T lymphocyte populations were larger in peripheral blood. In addition, dual color flow cytometry analysis revealed a high proportion of CD4⁺CD8⁺ cells in lymphocytes from peripheral blood and mesenteric lymph nodes in the Barodon-treated group, whereas a very small proportion of double-positive cells was observed in the control group. These results show that most CD4⁺ T lymphocytes coexpressed the CD8 antigen in pigs treated with Barodon. The larger double-positive population in peripheral blood and mesenteric lymph nodes of Barodon-treated pigs provides evidence of Barodon's effects on the porcine immune system. The difference in the double-positive population was greater in lymphoid organs, suggesting that Barodon may enhance antigenic stimulation in the immune tissues. Further studies using purified CD4⁺CD8⁺ double-positive cell populations will be necessary to elucidate the activity of Barodon more specifically.

Barodon's adjuvant properties have been demonstrated in pigs vaccinated against classical swine fever. Higher antibody titers, and greater proportions of MHC-class II⁺ and CD8⁺ immune cells and surface IgM⁺ B lymphocytes were observed in pigs treated with Barodon, compared to untreated controls.³⁴ Silica, among several candidate substances in Barodon, is likely responsible for these immuno-enhancing effects. Antonini et al³⁵ (2000) showed that subchronic silica exposure enhances respiratory defense mechanisms, for example, by increasing proportions of neutrophils, T lymphocytes, and natural killer cells, and increasing macrophage activation and pulmonary clearance of *Listeria monocytogenes*.

Other substances in Barodon, including minerals, may affect vital biological processes such as immune responses. When administered in an anionic aqueous solution such as Barodon, these substances may penetrate into body fluids more easily than similar products in powder formulations. This may contribute to Barodon's ability to enhance productivity and activation of immune responses in pigs. Although further studies are needed to elucidate the exact

mechanism of Barodon and its synergistic effect with vaccines or antibiotics in the porcine immune system, this study shows that Barodon may be a candidate immunostimulator and alternative to antimicrobial feed additives for improving productivity and host immune responses.

Implications

- Barodon has nonspecific immunostimulatory effects on porcine immune cells.
- Barodon may be an alternative to antimicrobials for improving productivity in the swine industry.

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Errata

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In Figure 1c, *P* values were incorrectly reported for weeks 11 and 13. The correct *P* values are .02 for week 11, and .05 for week 13.

Figure 1d was incorrectly reported, and the correct graph is provided. The proportions of non T/non B (N) cells were higher in the Barodon-treated group than in the control group at Study Weeks 3 (*P*=.02), 11 (*P*=.02), and 13 (*P*=.03), but not at Study Week 8.

Figure 1d: Proportions of non T/non B (N) cells in peripheral blood of pigs treated with Barodon (40 pigs) and in untreated controls (10 pigs).

