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Effects of 5-aminolevulinic acid on production of antibodies against classical swine fever live vaccine

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ABSTRACT

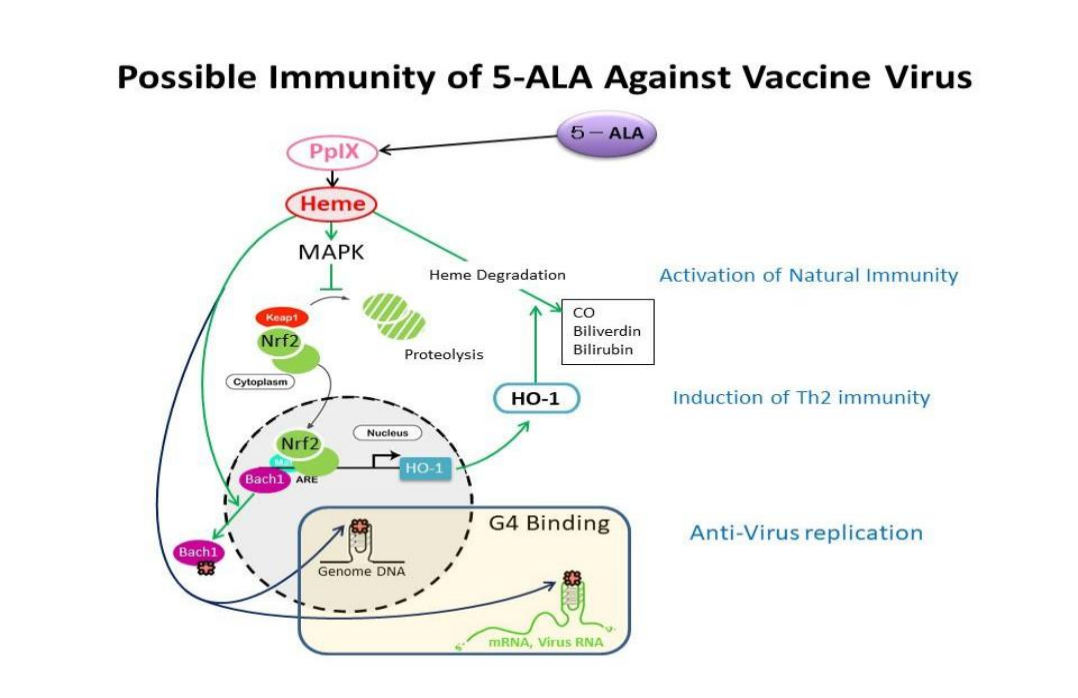
Introduction: The aim of this study was to examine the effects of a bioactive compound, 5-aminolevulinic acid (5-ALA), which has been reported to exhibit an immune-boosting effect, using a classical live swine fever (CSF) vaccine experimental model in pigs.

Methods: First, the effect of two different 5-ALA doses was evaluated by measuring the blood CSF viral load in male and female micro miniature pigs after vaccination with live vaccines. The CSF vaccine had a low inducing effect on antibody production in females, which improved after administration of 5-ALA by enhancing Th2 immunity as indicated by elevated interleukin-10 levels. Next, using male micro miniature pigs, the change in body weight was measured from the time before inoculation with the live vaccine to 28 days after inoculation, and the pattern of IgM and IgG antibody production after 5-ALA administration was examined.

Results: Preventive doses of 5-ALA enabled the continuous production of IgG antibodies at the same rate as found in control pigs not receiving 5-ALA; however, the switch to IgG production was delayed during 5-ALA treatment. Oral administration of 5-ALA kept the testing male pigs healthy, showing normal growth.

Conclusions: This suggested that the heme synthesis-promoting effects of 5-ALA simultaneously promoted the conversion of B cells into plasma cells.

Keywords: functional food, 5-ALA, human equivalent dose, pig, classical swine fever



INTRODUCTION

Five-aminolevulinic acid (5-ALA) is a natural amino acid found in mitochondria, which produces energy in all living organisms, including animals, humans, and plants. 5-ALA is converted to protoporphyrin IX (PPIX) in cells and forms a complex with iron in animals, subsequently forming heme, which is a component of hemoglobin, an oxygen carrier. In plants, magnesium is incorporated into chlorophyll, and 5-ALA is the source of chloroplasts that are essential for plant photosynthesis. Given this beneficial heme-forming ability, 5-ALA has been widely used for treating several human diseases and for metabolic improvement, such as hyperglycemia and sleep disorder in the form of a bioactive compound [1-2].

ALA was known to be effective against infectious diseases such as malaria due to its modification into PPIX in the body [3-4] and its ability to target the G4 protein [5]. Notably, the G4 protein complex was recently identified to have antiviral effects [6]. Further, 5-ALA has been demonstrated to be vital for the prevention and treatment of influenza in mouse models (SBI Pharma, Patent Application 2012-160999 WO2014013664A1) and in studies involving viruses infecting whiteleg shrimp (*Litopenaeus vannamei*) [7].

The world is currently in the throes of a pandemic caused by the novel coronavirus SARS-CoV-2, a positive-sense single-stranded RNA virus originating in bats [8]. Although a recent study indicated that swine are susceptible to low levels of SARS-CoV-2 viral infection [9], conducting in vivo challenge experiments with SARS-CoV-2 is not feasible. To determine the human equivalent dose of 5-ALA, in this study, a classical swine fever (CSF) live vaccine model was used to examine the effects of 5-ALA on antibody production, an important part of the infection establishment process.

This study can pave the way to facilitate better

understanding of the human equivalent dose of 5-ALA and of potential viral transmission between humans and pigs [9–11].

MATERIALS AND METHODS

This study was performed at an AAALAC international-approved facility. The animals were handled in accordance with the guidelines of Animal (Scientific Procedures) "Ministerial Ordinance on Good Laboratory Practice for Nonclinical Safety Studies of Drugs'' (Ordinance of Japan's Ministry of Health and Welfare No. 21 of March 26,1997, as last amended by the ordinance of Japan's Ministry of Health, Labor and Welfare No.114 of June 13, 2008). The experimental protocol was approved by the Animal Care and Use Committee of Hamri Co., Ltd. [approval no: 20-H050 & 20-H064].

Experimental animals and pharmacological agents: For the first experiment, 6 male and 6 female micro miniature pigs (MMP), aged 52–55 days and born at the same time in the same facility (Fuji Micra Co., Ltd, Shizuoka, Japan), were used. The males and females were each divided into three groups (n = 2 per group): the normal-feed group (control group), low-dose group (5 mg 5-ALA-HCl + 3.9 mg/kg sodium ferrous citrate [SFC]), and high-dose group (50 mg 5-ALA-HCL + 39 mg/kg SFC) (molar ratio of ALA:SFC = 1:0.25). The second experiment involved 8 male micro miniature pigs, aged 52–55 days, divided into two groups (n = 4 per group): the normal-feed group (control group) and the 5-ALA group (50 mg 5-ALA-HCl + 39 mg/kg SFC).

5-ALA and SFC were separately mixed into small amounts of normal feed, rolled into balls, and fed to the pigs before their morning meal, followed by their regular feed. This was done once daily, starting from four days before vaccine administration (Day –4) to 28 days after vaccine administration (Day 28).

CSF live vaccine: One vial of dried vaccine (for 20 animals) containing at least $10^{4.3}$ median tissue culture infectious disease dose ($TCID_{50}$) of the attenuated GPE^- strain of the CSF virus (seed) in guinea pig kidney cell culture was used. The included solvent solution was used to dissolve the dried vaccine, and 1 mL of vaccine solution was inoculated either subcutaneously or intramuscularly into the pigs (Figure 1a). All animals were inoculated once at 56–59 days of age (Day 0).

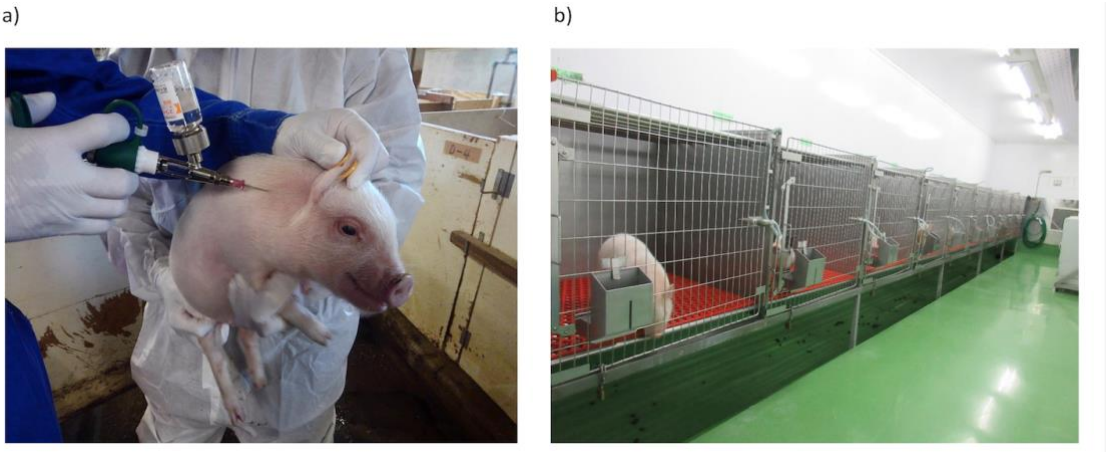


Figure 1. Microminiature pigs used in this study

a) MMPs aged 56-59 days were inoculated once by subcutaneous or intramuscular administration of CSF virus vaccine. **b)** MMPs were individually bred in a clean breeding environment at the AAALAC certified facilities.

Observation and testing: The change in body weight was measured from the time before inoculation with the live vaccine to 28 days after inoculation in the second experiment. An increase or decrease in body weight over time was observed relative to a standard growth curve (listing mean weights, +1 SD, and +2 SD) for microminiature pigs, as determined by the Shizuoka Prefecture Small and Medium Livestock Center (n = 23).

MMPs had been originally established in Japan as very small experimental miniature pigs [14]. MMPs were

fed using regular food of ATTACK KOBUTA produced by Marubeni Nisshin Feed Co., Ltd. (4-5-1 Muromachi, Nihonbashi, Chuo-ku, Tokyo, Japan). As the body weight of pigs is known to be dependent on diet, the feed is shown in Table 1. During the 5-ALA experiments, 70g/head of the feed was given twice a day (Total 140g/Day).

Table 1. Ingredients details of ATTAKBKOBUTA for testing MMPs

Ingredients Type	Ratio (%)	Name of the ingredients
Foodgrain	72	corn, precooked corn, milo, common wheat, brown rice, bread crumbs, (flour) soybean oil cake, rapeseed oil cake
plant-derived oil cake	22	rice bran, bran, (DDGS; corn distillers grains with soluble), (corn gluten feed)
plant-derived chaff and bran	2	fish meal, (mixed feed of pork & chicken)
animal-derived feed	1	molasses, animal fat, sweets crumb, calcium carbonate, salt, lactic acid bacterium, dried yeast cell wall (calcium phosphate)
others	3	

Antibody production in the blood was used as an index of infection establishment. After inoculation with the CSF live vaccine, the microminiature pigs were reared at Hamri Co., Ltd. (Ibaraki, Japan). Approximately 5 mL of blood was collected from the jugular veins of the tested pigs at 14, 28, and 42 days after inoculation (Figure 1b).

Anti-CSF antibody titers in the pigs' blood were quantified for both the IgM and IgG subtypes using enzyme-linked immunosorbent assay (ELISA) with CSF ELISA Kit II (JNC Corporation, Tokyo, Japan) according to the manufacturer instructions. IgM was inactivated (decomposed) upon mercaptoethanol treatment, and the level detected in the treated serum measured using the kit was taken as the IgG value. Absorbance was measured at a wavelength of 450 nm, and the S/P value of the test serum was calculated using the following formula:

$$S/P = [S(P) – S(N)]/[PC(P) – PC(N)]$$

where PC(P) is the average absorbance of positive-control serum in antigen-coated wells, PC(N) is the average absorbance of positive-control serum in antigen non-coated wells, S(P) is the absorbance of the test serum in antigen-coated wells, and S(N) is the absorbance of the test serum in antigen non-coated wells.

In addition, the S/P value of IgM was determined as described above by subtracting the S/P value of the serum treated with mercaptoethanol from the S/P value of the serum obtained before the treatment. Serum interleukin (IL)-4 and IL-10 levels were measured using an ELISA Kit (Cloud Clone CO., Wuhan, China). The blood CSF viral load was measured using the CSFV Real-time PCR kit (Kogene Biotech CO., Seoul, Korea).

RESULTS

Increase in the serum levels of IgM and IgG antibodies in response to the CSF live vaccine: In the first experiment, the CSF virus was detected on Day 3 or Day 14, and anti-CSF antibodies were detected on Day 28 and Day 42 after inoculation in the male control subjects (#4552 and #4556, Table 2). In contrast, in the female control subjects, CSF virus was not detected and the levels of the antibodies against CSF were very low (#4550 and #4551, Table 1). Administration of 50 mg of 5-ALA for one month drastically induced antibody production on Day 28 or Day 42 in the females (#4548 and #4549). In addition, an increase in serum IL-4 and IL-10 levels was observed in a few cases of 5-ALA-treated individuals (Table 2).

Table 2. Antibody production of micro mini pigs inoculated with classical swine fever (CSF) virus vaccine.

Male

			Control		5mg		50mg	
	Subject	No.	4552	4556	4553	4554	4555	4557
CSF virus Day3		−	+	+	+	+	+	
	Day14		+	−	−	−	−	−
	Day42		−	−	−	−	−	−
IgM + IgG	Day14		0.007	0.002	0.080	-0.003	-0.001	-0.001
	Day28		0.123	0.194	0.331	0.051	0.141	0.127
	Day42		0.369	0.450	0.291	0.291	0.179	0.228
IL-4[pg/mL]	Day3		<15	142	80	250	68	166
	Day14		106	121	178	160	26	186
	Day28		175	<15	85	296	131	158
	Day42		132	15	123	290	74	299
IL-10[pg/ml]	Day3		607	1675	768	2253	1599	1267
	Day14		1062	1016	682	1378	2136	816
	Day28		1289	359	1356	3017	2516	2083
	Day42		359	780	1289	3381	1142	1762

Female

		Control		5mg		50mg	
	Subject No.	4550	4551	4563	4564	4548	4549
CSF virus	Day3	−	−	+	−	−	+
	Day14	−	−	−	−	−	−
	Day28	−	−	−	−	−	−
IgM + IgG	Day14	0.004	0.002	0.003	0.000	0.000	0.016
	Day28	0.005	0.017	0.021	0.019	0.022	0.221
	Day42	0.003	0.112	0.028	0.027	0.351	0.391
IL-4[pg.mL]	Day3	92	27	<15	31	67	22
	Day14	<15	58	<15	166	48	<15
	Day28	<15	<15	<15	101	167	414
	Day42	67	227	139	410	75	<15
IL-10[pg/mL]	Day3	1131	317	1895	1857	645	707
	Day14	479	1478	976	2771	372	153
	Day28	230	816	1258	1119	2421	2913
	Day42	657	1729	1159	2832	1345	1017

CSF virus status (positive/negative) confirmed by RT-PCR; levels of anti-CSF virus antibodies and cytokines are shown for each individual in the first experiment.

Table 3. Levels of anti-CSF virus antibodies (IgM and IgG) for each individual in the second experiment

Male

		Control				50mg			
Subject No.		4529	4530	4534	4537	4528	4533	4535	4536
IgM	Day14	-0.031	-0.014	-0.003	-0.001	-0.036	0.003	-0.005	-0.027
	Day28	0.968	0.355	0.341	0.559	0.847	0.627	0.386	0.861
	Day42	0.159	0.147	0.302	0.330	0.276	0.233	0.313	0.353
IgG	Day14	0.098	0.016	0.012	0.005	0.047	0.003	0.009	0.017
	Day28	0.030	0.115	0.559	0.329	-0.005	-0.005	0.152	0.000
	Day42	0.558	0.161	0.327	0.225	0.360	0.152	0.177	0.404

As described above, in the male control group, the CSF virus was detected 14 days after vaccination, suggesting that males are more susceptible to the virus than females. In addition, both IL-4 and IL-10 levels tended to be higher in the 5-ALA administration groups (#4554, #4555, and #4557, Table 2). Therefore, IgG and IgM were separately examined for antibody production in males in the next experiment.

Anti-CSF IgM was not detected in either the control or 5-ALA group at Day 14, although it was detected at the same level in both groups at Day 28 (Table 3). The fact that antibody-based immunity had been elicited in all animals indicated that the CSF live vaccine had successfully established a CSF infection. By contrast, anti-CSF IgG levels followed a different pattern (Table 3): anti-CSF IgG was not detected in either group at Day 14, and its levels increased over time in the control group but not in the 5-ALA group.

Changes in weight: Changes in the body weight of the animals in both the 5-ALA and control groups in the second experiment are shown in Figure 2b. In terms of the growth rate, 3 of 4 pigs in the 5-ALA group (#4533, #4535, #4536) were below the +1 SD curve prior to the administration of 5-ALA (at 45–48 days of age; Day –11); however, after administration of 5-ALA (at 66–69 days and at 84–87 days of age; Day 10 and Day 28, respectively), the data points were above the +1 SD curve. In other words, these three pigs grew faster, showing a growth rate higher than the standard growth rate. A similarly accelerated growth rate pattern was observed for only one out of four pigs in the control group (#4530).

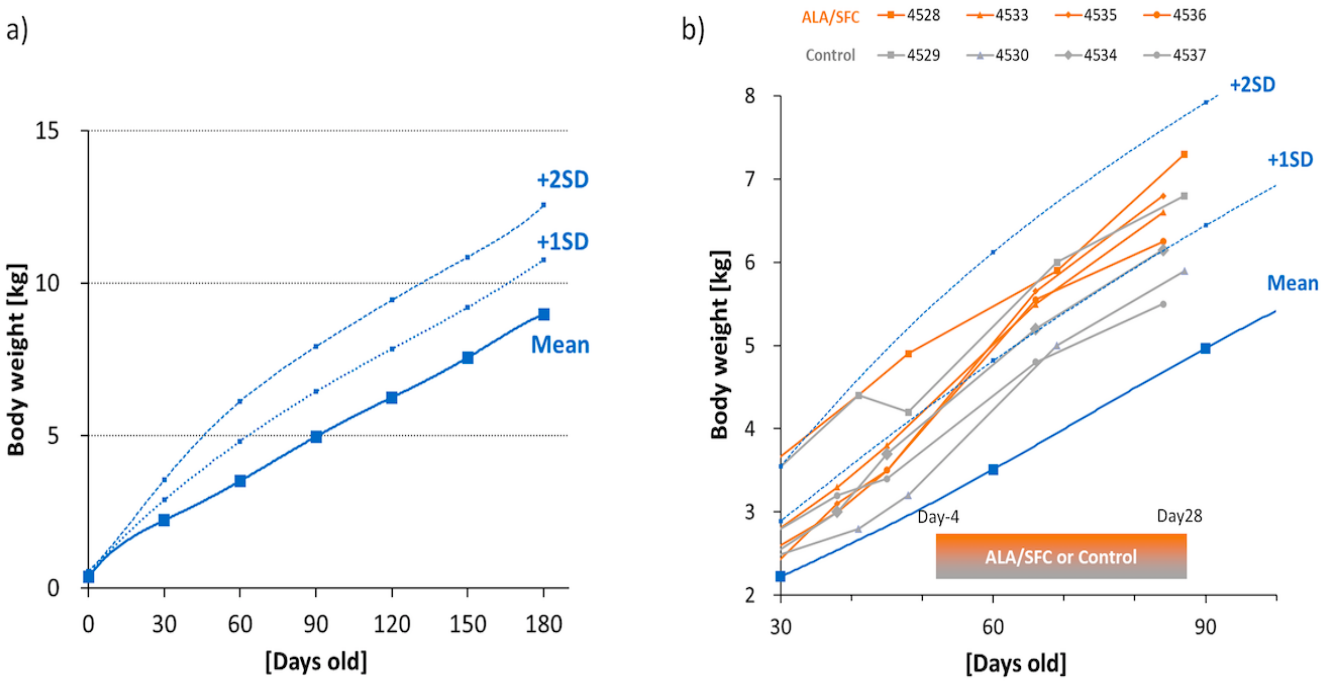


Figure 2. Body weight gain of MMPs

a) The standard growth curve of MMPs. The body weight of MMP (n=23) bred at Shizuoka Prefectural Research Institute of Animal Industry, Swine & Poultry Research Center was measured every 30 days. The average value, +1SD and +2SD values were plotted (blue). **b)** The body weight of each MMP in the ALA/SFC administration group (#4528, #4533, #4535, #4536, orange) and the control group (#4529, #4530, #4534, #4537, gray) was plotted on the standard curve (blue). CSF vaccines were administered at Day 0 (when 56-59 days old). ALA/SFC or control was administered once a day during Day-4 to Day28.

The mean daily increase in the body weight of each animal is shown in Table 4. The mean rate of body weight increase for four pigs in the control group was 0.077 kg/day for the first half of the administration period (Day 11 to Day 10) and was 0.049 kg/day for the second half (Day 10 to Day 28). In the 5-ALA group, the mean rate of body weight increase for the first half of the administration period was 0.082 kg/day and that for the second half was 0.064 kg/day. Therefore, the rates of body weight increase in the 5-ALA group were 106% and 130% of those in the control group during the first and second halves of the administration period, respectively. This indicated that administration of 5-ALA accelerated the increase in body weight.

Table 4. Average daily weight gain for each pig

		Average daily weight gain[kg]		
		Day-11 to Day10	Day10 to Day28	Day-11 to Day28
Control	4529	0.086	0.044	0.067
	4530	0.086	0.050	0.069
	4534	0.071	0.053	0.063
	4537	0.067	0.039	0.054
	Mean	0.077	0.047	0.063
		100%	***100%***	***100%***
ALA/SFC	4528	0.048	0.078	0.062
	4533	0.081	0.061	0.072
	4535	0.102	0.064	0.085
	4536	0.098	0.039	0.071
	Mean	0.082	0.060	0.072
		106%	***130%***	***114%***

Day 0: Vaccination
Italics indicate the ratio of the mean value of the ALA/SFC administration group when the average value of the control group was set to 100%.

DISCUSSION

The current study was conducted in an effort to identify health foods capable of preventing viral infections. Bach2 has been shown to regulate the connected processes of heme transport, degradation, and processing of the degradation byproducts, besides its effects on natural and acquired immunity [12], thereby suggesting the possibility of developing a new cooperative system linking metabolism and immunity. Based on this background, 5-ALA, which is involved in heme synthesis, could advantageously promote viral elimination. In an effort to predict the infection-preventive effects of 5-ALA in humans, microminiature pigs were used in this study, since this animal model can be used to easily extrapolate human equivalent doses [13-14]. Using needle-inoculation of a CSF live vaccine, the possibility of 5-ALA manifesting antiviral properties was investigated by examining its effects on antibody production, which forms an important part of the infection establishment process.

In an effort to simulate its infection-preventive use in humans, 5-ALA administration was initiated prior to vaccine inoculation in this study. In the first experiment, the response to the live CSF vaccine was found to be different in males and females. In the female control group (5-ALA non-administered group), the CSF virus was not detected in the blood, and none of the control females showed antibody production. Surprisingly, when females inoculated with live CSF vaccines received 5-ALA in their feed for one month, antibody production was successfully induced. This tendency continued for up to two weeks after the end of the 5-ALA treatment. This immune response corresponded with an increase in IL-4 and IL-10 levels.

In the male control group, CSF virus was detected even 14 days after vaccination, suggesting that males are more susceptible to the virus than females. However, in contrast to the females, 5-ALA administration in males did not show an apparent effect on antibody production. Nevertheless, the levels of both IL-4 and IL-10 tended to be higher in the 5-ALA administration groups. Therefore, IgG and IgM were separately examined for antibody production in males in the next experiment.

Surprisingly, although the pigs from the control group produced both IgM and IgG antibodies, those from the 5-ALA group produced only IgM antibodies. This suggested that even when live virus injection established infection in the pigs, the switch from IgM to IgG did not occur. The phenomenon of class switching is as follows: naive B cells express IgM antibodies on the surface of their cell membranes; upon activation, a DNA recombination reaction known as “class switching” occurs in the constant region of globulin genes, causing B cells to express either IgG or IgA. There are other cells that do not undergo class-switch recombination, and rather differentiate into plasma cells, which then secrete IgM antibodies. Finally, identification of mitochondrial reactive oxygen species (mROS) as a heme synthesis regulator can further enhance the understanding of mechanisms regulating heme homeostasis and cell-fate determination after B-cell activation [15]. Moreover, heme can bind to Bach2, a transcription factor essential for humoral immunity, including antibody class switching.

Watanabe-Masui et al. [16] reported that heme inhibited the DNA-binding activity of Bach2 in vitro and reduced its half-life in B cells. Further, when added to B-cell primary cultures, heme enhanced the transcription of Blimp-1, the master regulator of plasma cells, and skewed plasma cell differentiation toward the IgM isotype, decreasing the IgG levels in parallel [16].

Thus, it is possible that administration of 5-ALA promoted heme synthesis, which induced the differentiation of B cells into plasma cells, thereby selectively increasing the levels of IgM antibodies only. In humans, the Nef auxiliary protein of HIV-1 has been reported to inhibit Ig class-switching [17]. Recently, an increased level of antibody production in patients infected with SARS-CoV-2 has been reported, but changes in antibody features and their mechanisms have not yet been fully elucidated [18].

This study utilized the increase in antibody levels in response to the administration of the live vaccine to demonstrate infection establishment. Since the administration of 5-ALA to pigs has been shown to advantageously affect growth and protect against infection [19], the growth of pigs that did and did not receive 5-ALA during this trial were compared with reference to a standard growth curve. Compared to the control group, the 5-ALA group demonstrated a slowly accelerating increase in weight. This result aligned with results of previous reports, indicating that 5-ALA positively affected the growth and immunocompetence of other species of livestock [20–22].

Healthy individuals are believed to benefit from the daily use of 5-ALA, which could prevent infection from several aspects. 5-ALA can aid in replacing functionally degraded heme proteins with new ones, thereby helping the maintenance of various redox reactions regulated by heme at a higher level. Infectious diseases often affect many patients in subclinical ways; if the ability to synthesize heme is maintained at a high level and the immune strength is also maintained properly, the body will be quite capable of eliminating viruses naturally, and the preventive use of 5-ALA would boost this capacity further. By contrast, if the ability of the body to synthesize heme drops below a certain level, viruses cannot be eliminated, thereby requiring more heme, eventually pulling the body into a vicious cycle of heme deficiency. The low body-temperature state, often observed in the early stages of viral infection (before fever onset), is caused by heme deficiencies owing to limited thermogenesis, and febrile symptoms may indeed be the result of the imbalanced distribution of body resources for heat production as an emergency response to cause viral elimination. It is worthwhile saying that 5-ALA is safe and effective for antibody production under administration of CSF live vaccine in pig. Although 5-ALA has been recently shown to be effective against SARS-CoV-2 in vitro [23], its efficacy for COVID-19 patients should be investigated according to the adequate clinical protocol.

CONCLUSION

This study is a preliminary exploration of the antiviral and preventive effects of 5-ALA; a CSF live-vaccine inoculation model was used, and post-inoculation antibody production was examined to determine the establishment of infection. Although this model differs from experimental infection with the novel coronavirus SARS-CoV-2, the results indirectly reflect the effects of vaccine inoculation on healthy individuals receiving 5-ALA, which is considered to be a health-promoting compound.

Although the measurement of IgM and IgG antibody production did reveal the establishment of infection, the administration of 5-ALA promoted heme synthesis, halting class switching to IgG, thereby inducing the reversal of B cells to plasma cells, ultimately causing elimination of the virus. Recent reports have indicated angiotensin converting enzyme 2 (ACE2) as the receptor through which SARS-CoV-2 infiltrates the host cells [24–26]. PPIX is believed to adhere to ACE2 and prevent the binding of SARS-CoV-2 spike proteins [27]; high-dose administration of ALA, which causes PPIX production, has demonstrated remarkable efficiency in this regard. Thus, this suggests that 5-ALA might have the ability to prevent other infections beyond CSF virus infection.

It is necessary to determine the human equivalent dose of health foods such as 5-ALA by evaluating their secondary pharmacological effects other than the antiviral effect using experimental pigs, which are similar to humans in terms of body size and metabolism [28]. Although 5-ALA was shown to be effective against SARS-CoV-2 in vitro [23], its efficacy for COVID-19 patients should be investigated further.

List of Abbreviations: ACE2: angiotensin converting enzyme 2, CSF: classical live swine fever, HED: Human Equivalent Dose, MMP: micro miniature pigs, mROS: mitochondrial reactive oxygen species, PPIX: protoporphyrin IX, 5-ALA: 5-aminolevulinic acid.

Authors Contribution: Study concept and design, performing experiments, analysis and interpretation of data, drafting of the manuscript, revision of the manuscript and statistical analysis: Eiji Kobayashi

Conflict of Interest: Eiji Kobayashi is a medical advisor of Neophama Japan Co., Ltd.

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