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Supplement of Conjugated Linoleic Acid Increases Neutrophil Phagocytosis in Pigs

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Abstract : To examine the *in vivo* immunostimulating effect of conjugated linoleic acid (CLA) in pigs, the change of peripheral blood cells and the phagocytic response of phagocytes were evaluated. Spayed male pigs, 80 kg of average body weight, fed a diet containing either 0.5% 10t-12c CLA or 0.5% CLA mixture (mostly 9c-11t CLA and 10t-12c CLA) for 4 weeks. The change of blood cell values (PCV, WBC, differential count of WBC) and the phagocytic activities of phagocytes were evaluated on week 0, 2, 4, and 5, respectively. There were no change in the PCV values regardless of CLA supplement. The number of WBC, especially neutrophils, in pigs fed a diet with CLA was significantly increased ($p < 0.05$ to 0.01) when compared with control pigs fed a diet without CLA. The phagocytosis of peripheral blood mononuclear cell (MNC) and peripheral blood polymorphonuclear cells (PMN) were analyzed by a flow cytometry system. There was no change in the phagocytic activity of MNC and monocyte-rich cells regardless of CLA supplement. However, the phagocytic activity of PMN composed by approximately 95% neutrophils was remarkably increased ($p < 0.05$ to 0.01) on week 2, 4, and 5 as compared wth control pigs. These results suggested that supplement of CLA into pigs induces the increase of neutrophil number and the enhancement of neutrophil phagocytosis.

Key words **:** conjugated linoleic acid (CLA), neutrophil, phagocytosis, pig

Introduction

A wide variety of antibacterial agents, mainly antibiotics, are employed for the prevention and treatment of infection in livestock. But, the administration of these drugs causes various problems such as residue and emergence of bacterial resistance. In recent years, the depressed immune function attributable to stress in swine has caused a marked increase in the incidence of opportunistic infections such as porcine reproductive and respiratory syndrome virus (PRRSV)[7] and porcine circovirus (PCV)[22] or complicated infections such as *Pasteurella multocida*[2] and *Actinobacillus suis*[10] which are difficult to treat with antimicrobial agents alone. Bacterial pneumonia caused by *Pseudomonas aeruginosa* and chronic respiratory bacterial infections are common in granulocytopenic patients[4,13,32]. These diseased animals also have recurrent infections because of the reduced number and function of neutrophils which mediate an early stage of host defense[4,12,26]. Therefore, a major thrust in the field of antimicrobial therapy is to focus on the improvement of host defense mechanisms. Application of immunostimulants seems promising as a measure for the prevention or treatment of various infections.

Conjugated linoleic acid (CLA) isomers include both *cis-cis*, *cis-trans* and *trans-trans* geometry with double bonds at 9 and 11, 10 and 12 or 11 and 13, 7 and 9, 12 and 14 or 8 and 10. These isomers are enriched in the tissues of ruminant animals and in dairy products[14]. The immune system also appears to be affected by CLA[29]. Dietary CLA has been shown to increase immunoglobulin production in rat spleen lymphocytes[35] and to reduce antigen-induced histamine and prostaglandin (PG)E_2 release from sensitized guinea pig trachea[39]. CLA in physiological concentration inhibits proliferation of several human cancer cell lines *in vitro*[30,33,34]. The 9c-11t CLA and 10t-12c CLA in numerous isomers are known to have biological activity[8]. The 10t-12c CLA alters lymphocyte blastogenesis[11]. Mixtures of CLA isomers (mostly 9c-11t CLA and 10t-12c CLA) have been shown to enhance the immune system and reduce the catabolic effects of immune stimulation[9]. More recently, we reported that CLA isomers such as 10t-12c CLA, 9c-11t CLA and CLA mixture have an enhancing effect on chemotaxis of porcine peripheral blood polymorphonuclear cells (PMN), which may be mediated through interleukin (IL)-8-like factor produced by CLA-stimulated mononuclear cells (MNC)[17]. It was also assumed that MNC activated by CLA isomers may release many factors associated with host defense and immune response.

In the present study, CLA-supplemented diets were fed to pigs and its effect on the change of peripheral blood cells and neutrophil phagocytosis was examined.

Materials and Methods

Animals

Spayed male crossbred pigs of 100-day-old age, 80 kg of average body weight, were used as blood donors. All pigs were housed in temperature controlled room with an alternating 12 hrs light/dark cycle and fed on commercial diet and tap water.

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Experimental Protocols

The pigs were divided into three groups: 1) control fed a diet without CLA, 2) pigs fed a diet with 0.5% 10t-12c CLA (purity 91%; H&K Bioteck, Korea), and 3) pigs fed a diet with 0.5% CLA mixture (43% 10t-12c and 45% 9c-11t CLA; H&K Bioteck, Korea). Each group consisted of 5 pigs, respectively. The pigs were freely fed on a diet mixed with CLA at 0.5% ratio of CLA/diet for 4 weeks. After the supplement of CLA, pigs were also freely fed on a diet without CLA for 1 week. Peripheral blood samples were collected from anterior venae cava on weeks 0, 2, 4, and 5 to examine the peripheral blood cell values and phagocytic activity of phagocytes.

Blood cell count

Peripheral blood samples were collected in heparinized tube from anterior venae cava. Packed cell volume (PCV) and white blood cell (WBC) count were measured using manual method within 30 minutes after blood collection.

MNC and PMN isolation

Peripheral blood was collected in heparinized tube from anterior venae cava. Blood diluted with the equal volume of phosphate-buffered saline (PBS) at pH 7.6 was layered 1:1 on Ficoll-hypaque solution (specific gravity, 1.080; Pharmacia Inc., USA) and centrifuged at 400×g for 40 minutes at room temperature. The MNC in interface between PBS plus plasma and Ficoll-hypaque solution was harvested and washed 3 times with PBS. The PMN was obtained from layer of erythrocyte sediment after collection of MNC. One ml of the upper part of the erythrocytes was mixed with 10 ml of 1.5% dextran (molecular weight, 200,000; Wako Ltd, Japan) in PBS and allowed to sediment for 60 minutes. The residual erythrocytes were lysed by treatment with 0.83% NH4Cl solution for 5 minutes at 37°C and washed 3 times with PBS. The purity of neutrophils in the final PMN suspension was approximately 95% when determined in Wright- and Giemsa-stained smears. The viability of MNC and PMN determined by a trypan blue dye exclusion test was always more than 98%. All cells were resuspended in RPMI 1640 (Gibco Co., USA) supplemented with 2 mM L-glutamine, 0.02 mg/ml of gentamicin and 5% fetal bovine serum (Gibco Co.,) and finally adjusted to 1×10^6 cells/ml.

Phagocytosis assay

The phagocytosis activity of PMN and MNC was determined as described previously[19,41]. The cells at density of 1×10^6 cells/ml were put into each well of a 24 well-plate (Becton Dickinson Labware, USA). Twenty microliter of 1×10^9 particles/ml of fluorescein isothiocyanate (FITC)-labelled latex beads (size: 2.0 μm; Polysciences Inc., USA) were added, and then incubated for 1 hr at 37°C under 5% CO_2-humidified atmosphere. The incubated cells were harvested gently by slow pipetting, centrifuged at 400×g for 4 minutes, and washed 3 times with PBS containing 3 mM EDTA-2Na. The latex bead-phagocytized cells per total 10,000 cells in MNC and PMN were immediately analyzed by a flow cytometry (FACS Calibur, Becton Dickinson Immunocytometry Systems, USA). To use the monocyte-rich cells in flowcytometric cytography, they were fractioned by cell size of MNC from dot plot profile of flowcytometric cytography. The phagocytized cells per total 5,000 cells in monocyte-rich fraction were measured. Results were expressed as percentage of absolute phagocytic activity.

Data analysis

The Student's t-test was used for statistical significance determinations. All data were expressed as mean ± standard error (SE).

Results

PCV values

There were no change in the PCV values regardless of the supplement of 10t-12c CLA or CLA mixture (Fig 1). The PCV values were within normal ranges.

Changes in WBC number

The number of WBC in pigs fed 10t-12c CLA was significantly increased ($p < 0.01$) when compared with the control on weeks 2, 4 and 5 (Fig 2). The WBC in CLA mixture-fed pigs on week 4 was also significantly increased ($p < 0.01$) when compared with the control. On week 5, the pigs showed a greater number although there were not significant when compared with the control

Differential counts of WBC

The differential counts of WBC showed no significant differences except for neutrophils between control and CLA-supplemented groups. The neutrophil value in pigs fed with either 10t-12c CLA or CLA mixture was significantly ele-

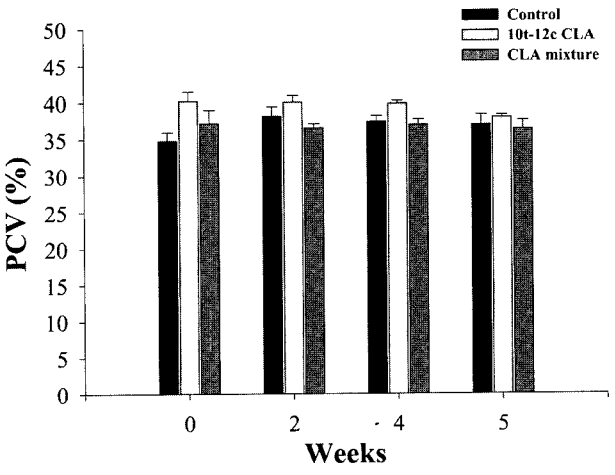

Fig 1. The change of PCV value (%) in pigs fed 10t-12c CLA or CLA mixture. The data represent mean± SE (n=5).

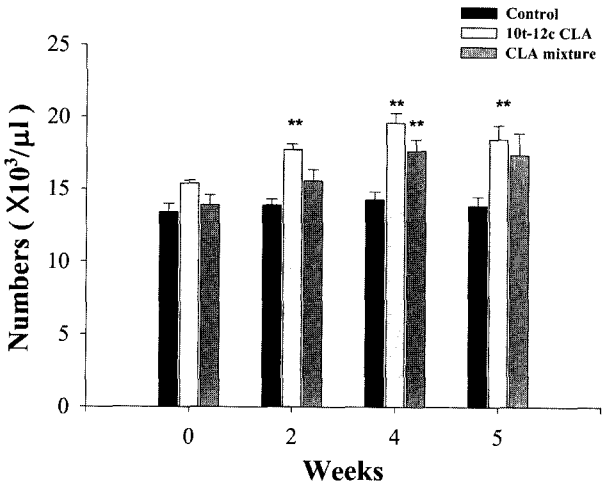

Fig 2. The change of WBC number in pigs fed 10t-12c CLA or CLA mixture. The represent mean± SE (n=5). *P< 0.05, **P< 0.01 compared to control.

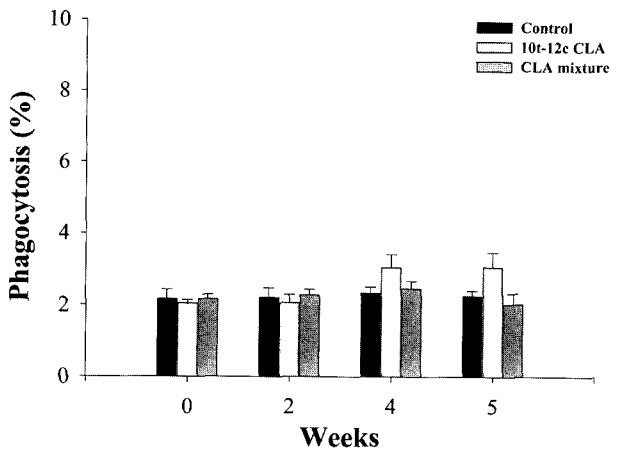

Fig 3. Phagocytic activity of MNC in pigs fed 10t-12c CLA or CLA mixture. The data represent mean± SE (n=5).

vated ($p < 0.05$ to 0.01) on weeks 2, 4, and 5 as compared with the control, and the greatest increase was observed on week 4 (Table 1).

Phagocytic activity of MNC and monocyte-rich cells

As shown in Fig 3, there was no change in the phagocytic activity of MNC in pigs supplemented with either 10t-12c CLA or CLA mixture when compared with control pigs. Also, the phagocytic activity of monocyte-rich cells showed no significant change (Fig 4).

Phagocytic activity of PMN

As shown in Fig 5, pigs fed 10t-12c CLA had significantly greater phagocytic activity of PMN ($p < 0.05$) on weeks 4 and 5, but on week 2, although they showed no sig-

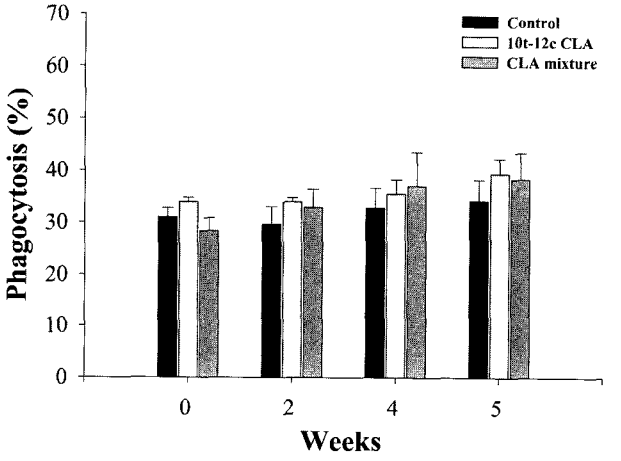

Fig 4. Phagocytic activity of monocyte-rich cells in pigs fed 10t-12c CLA or CLA mixture. The data represent mean± SE (n=5).

Table 1. The differential counts of WBC ($\times 10^3/\mu l$) in pig fed either 10t-12c CLA or CLA mixture

Week	Cell typeGroup	Neutrophil	Eosinophil	Basophil	Monocyte	Lymphocyte
0	Control	4.2± 0.31	0.2± 0.07	0	1.2± 0.12	7.8± 0.38
	10t-12c CLA	4.5± 0.10	0.3± 0.06	0	1.4± 0.08	9.2± 0.18
	CLA mixture	4.2± 0.18	0.3± 0.05	0	1.3± 0.08	8.3± 0.50
2	Control	4.4± 0.26	0.3± 0.05	0	1.4± 0.12	8.5± 0.69
	10t-12c CLA	6.1± 0.12**	0.2± 0.04	0	1.4± 0.10	10.1± 0.17
	CLA mixture	5.5± 0.36*	0.2± 0.02	0	1.2± 0.08	8.7± 0.49
4	Control	4.4± 0.25	0.2± 0.03	0	1.3± 0.15	8.3± 0.32
	10t-12c CLA	7.5± 0.29**	0.1± 0.04	0	1.3± 0.12	10.7± 0.31
	CLA mixture	6.7± 0.34**	0.1± 0.04	0	1.3± 0.11	9.5± 0.56
5	Control	4.4± 0.28	0.2± 0.02	0	1.3± 0.11	8.0± 0.35
	10t-12c CLA	6.7± 0.27**	0.1± 0.04	0	1.3± 0.10	10.4± 0.61
	CLA mixture	6.4± 0.62*	0.1± 0.04	0	1.3± 0.16	9.6± 0.84

The data represent mean ± SE (n=5)
*P < 0.05 , **P < 0.01 compared to control

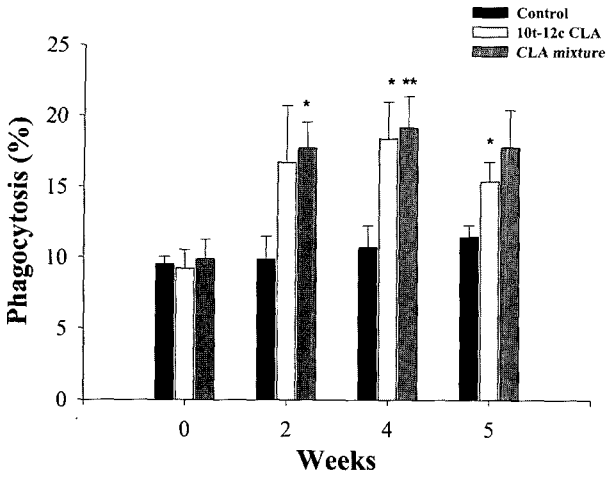

Fig 5. Phagocytic activity of PMN in pigs fed 10t-12c CLA or CLA mixture. The data represent mean± SE (n=5). *P<0.05, **P<0.01 compared to control

nificance as compared with the control pigs, the phaogocytic activity was very high. The phagocytic activity of PMN in pigs supplemented with CLA mixture was also remarkably increased ($p < 0.05$ to 0.01) on weeks 2 and 4 as compared with that of the control and then slightly decreased on week 5, but it was still very high.

Discussion

The results of this study indicated that CLA did not appear to influence the erythropoiesis of pigs since there were no change in PCV values regardless of CLA supplementation, alike in EWD on the hematopoiesis and growth of erythrocytes of cat[40]. But supplement of a diet with 0.5% CLA to pigs increased the number of WBC, especially neutrophils, suggesting that CLA has an effect in stimulating hematopoiesis granulocyte series. It is possible that the stimulatory effect of CLA for granulcytes might be due to some factors contained in CLA components. Alternatively, it may be due to an indirect action of substances taken up and metabolized from CLA. However, tumor necrosis factor (TNF)-α as one of the hematopoietic cytokines has been shown to act as a positive regulator of myeloid cell proliferation and differentiation[15,16,25]. Furthermore, the effects of TNF-α can be either directly or indirectly mediated by inducting other cells to induce the production of a variety of hematopoietic growth factors including IL-1[27], IL-6[6], macrophage-colony sti-mulating factor (M-CSF)[5,28], granulocyte-colony stimulating factor (G-CSF)-1[21] and granulocyte macrophage-colony stimulating factor (GM-CSF)[24]. Hematopoietic growth factors stimulated the growth and proliferation of granulocytic progenitor cells in the hematopoietic system[32]. CSF-1 strongly promotes the survival, function, proliferation, and differentiation of phagocytes[36]. In this regard, it was assumed that CLA stimulates granulocytes directly or indirectly.

The present results also revealed that the phagocytic activity of MNC and monocyte-rich cells by CLA supplement was not effective. This may be associated with fractions in MNC population. Since the fraction of MNC isolated from porcine peripheral blood was composed of both approximately 13% monocytes and 87% lymphocytes.

The supplement of CLA to pigs elicited a clear phagocytic enhancement of peripheral blood PMN composed by approximately 95% neutrophils. This finding was consistent with that of previous studies on phagocytosis for neutrophils in cats[40,41] or rats[19] receiving egg white derivatives (EWD), one of immunostimulators. The enhancing effect of EWD on phagocytosis of periphral blood neutrophil in these models was mediated by soluble products released from peripheral blood MNC treated with EWD[19,40,41]. In recent study, the phagocytic activity for PMN was enhanced by culture supernatant from MNC exposed to 9c-11t and 10t-12c CLA isomers[18]. This fact is suggesting that CLA isomers stimulate MNC to release active humoral factor(s), which may be an important mechanism for the increase of neutrophil phagocytosis. A variety of cytokines such as IL-1, IL-2, IL-8, TNF-α were produced by activated monocyte-macrophages and lymphocytes. These cytokines play an important role in the phagocytosis or chemotaxis of phagocytes. The representative phagocytosis-promoting factors produced by activated MNC are known as IL-1 and TNF-α[1,37]. It was suggested that the phagocytosis-promoting factor, which is produced by MNC in response to EWD, will be associated with the humoral factor of 16 to 18 kDa[41]. Also, the phagocytic activity for porcine PMN in culture supernatant from MNC exposed to EWD was identified as TNF-α with molecular weight of 16 to 18 kDa but not IL-1[20]. TNF-α has been shown to be associated with several biological effects including PMN activation and immune regulation[3,23]. It augmented the phagocytosis and antibody-dependent cytotoxic effector function of polymorphonuclear leukocytes[31,38]. It is, therefore, suggested that the enhancement of phagocytic activity of neutrophil in pigs fed CLA may be due to soluble products, such as TNF-α, which are involved in the nonspecific immunity of host defense.

This study strongly suggested that supplement of a diet containing CLA isomer to pigs induces the increase of number and phagocytosis of neutrophils, thus reinforcing the host defense mechanisms. For the clinical application, the supplement of CLA will be able to enhance the nonspecific immunity of peripheral blood WBC.

Conclusion

The in vivo immunoenhancing effect of CLA isomers (10t-12c CLA and CLA mixture) on the change of peripheral blood cells and the phagocytic activity of phagocytes was examined. There were no changes in the PCV values and differential counts of WBC except for neutrophils regardless of the supplement of CLA isomers. However, the number of WBC, especially neutrophils, were increased by CLA supplement.

In addition, the phagocytic activity of neutrophils of pigs fed a diet with CLA was remarkably increased. This study suggested that supplement of CLA to pigs induces the increase of number and phagocytic activity of peripheral blood neutrophils.

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Conjugated Linoleic Acid를 급여한 돼지의 호중구 탐식능 증강

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요 약 : CLA를 급여시킨 돼지에 있어 말초 혈액 세포의 변화와 탐식세포의 탐식반응을 조사하였다. 100일령, 평균체중 80 kg인 거세 수돼지에게 10t-12c CLA와 CLA mixture를 각각 사료 중에 5% 첨가하여 총 4주간에 걸쳐 자유급여 시켰다. 급여 전 (0주), 급여 2주후, 4주후 그리고 급여 중지 후인 5주차에 각각 혈액세포 수치(PCV, 백혈구수 및 백혈구 감별계산치)의 변화와 탐식세포의 탐식활성을 측정하였다. CLA의 급여 따른 PCV의 수치에 있어서는 아무런 변화가 없었다. 그러나 10t-12c CLA 또는 CLA mixture가 함유된 사료를 급여한 돼지에 있어서 CLA를 급여하지 않은 돼지에 비해 백혈구수 특히 호중구 수가 현저히 증가하였다. 말초혈액 단핵구세포(mononuclear cells; MNC)와 다형핵백혈구(polymorphonuclear cells; PMN)의 탐식능를 유세포분석기로 분석한 결과, 10t-12c CLA 또는 CLA mixture 급여와 상관없이 MNC와 단구세포들(monocyte-rich cells)의 탐식능에는 변화가 없었다. 그러나 PMN의 탐식활성은 CLA 무급여 대조돼지들에 비해 CLA 급여군은 2주에서 5주차에 걸쳐 증가된 탐식능를 나타내었다. 이상의 결과로부터 돼지에 CLA 급여는 호중구수 증가와 호중구 탐식능 증가를 유도할 수 있음을 제시하였으며 향후 생체방어 증강을 위해 CLA를 임상적으로 투여할 수 있을 것으로 보인다.

주요어 : Conjugated linoleic acid, 호중구, 탐식능, 돼지