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Zinc Increases Chemotactic Activity of Porcine Peripheral Blood Polymorphonuclear Cells

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Abstract : Zinc is necessary for normal functions in the immune system. The objective of the study is to examine the effect of zinc on the chemotactic activity of porcine peripheral blood polymorphonuclear cells (PMNs). A modified Boyden chamber was used to determine the directional migration distance of PMNs. Various concentrations of zinc showed no chemotactic activity to PMNs. However, culture supernatant from peripheral blood mononuclear cells (PBMCs) treated with zinc remarkably increased the chemotactic activity of PMNs when compared with culture supernatant from PBMCs treated without zinc. Culture supernatant from PBMCs treated without zinc also increased the migration distance of PMNs relative to vehicle control (medium alone). Increasing effect in chemotactic activity of PMNs by culture supernatant from PBMCs treated with zinc was inhibited by treatment of porcine anti-interleukin (IL)-8 polyclonal antibody (pAb). This effect was not affected by heat treatment (4-85°C). This corresponded with heat stable physical characteristics of IL-8. These results suggest that zinc can upregulate the chemotaxis of PMNs, which is primary mediated by IL-8 chemotactic factor released from PBMCs treated with zinc.

Key words **:** zinc, chemotaxis, peripheral blood mononuclear cells, peripheral blood polymorphonuclear cells, IL-8, pig.

Introduction

Peripheral blood polymorphonuclear cells (PMNs) play important roles in innate immunity. Neutrophils kill foreign bacteria through phagocytosis, releasing lysosomal enzymes from granules, generating reactive oxygen metabolites through respiratory burst and neutrophil extracellular traps (NET) formation (5,6). In order to successfully defense body from invader, neutrophils must migrate from the circulating blood to infected tissue. This is mediated by substances such as bacterially produced n-formyl-methionine-leucine-phenylalanine (fMLP), complement-derived C5a, leukotriene (LT) B4 and interleukin (IL)-8 (13,18). Various cytokines including interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor (TNF)-α secreted by activated monocytes, tissue macrophages and peripheral blood mononuclear cells (PBMCs) can also modulate defense against bacterial infections (16,27).

Zinc is trace element and necessary to maintain the physiological homeostasis in plasma pool. It was studied that pharmacological supplementation of zinc can revitalize impaired immune functions (24). Similarly, optimal supplementations of zinc decreased infection incidence and restored impaired immune response (19). It was reported that stimulation of THP-1 cells with zinc increases activation of NF-κB and release of cytokine IL-8 (7). Moreover, zinc ion can amplify cytokine production in LPS stimulated human PBMCs (28). On the other hand, Zinc depletion decreased all function of monocytes and phagocytic capacity of neutrophils (12). It was reported that zinc deficiency reduces IL-2 production of T-cells and causes impaired phagocytosis capacity of neutrophils and macrophages (1,4,15). It was known that zinc can increase phagocytic capacity of monocytes and PMNs (17). A previous study suggested that abnormally high concentration of zinc (> 500 μM) can directly induce chemotaxis of PMNs (11). However, there are no studies for physiological concentration of zinc on the chemotaxis of PMNs.

The aim of the present study is to investigate the effect of zinc on the chemotaxis of porcine PMNs. For this purpose, we examined the direct effect of zinc on chemotaxis of PMNs. We also examined the migration distances of PMNs by culture supernatant from zinc-treated PBMCs. We tested neutralization effect of anti-recombinant porcine IL-8 antibody to examine whether chemotactic effect of PMNs is mediated by IL-8 chemotactic factor.

Material and Methods

Reagents

Zinc sulfate (Fluka Chemie AG, Buchs, Switzerland) was commercially purchased. To prepare stock solution, it was diluted to a final concentration of 2 mM. And it stored at 4°C until before use. Goat anti-recombinant porcine IL-8 polyclonal antibody (IgG) (R&D systems Inc., Minneapolis, MN, USA), fetal bovine serum (FBS) (Gibco Company, Grand Island, NY, USA), rabbit anti-recombinant mouse IL-6 polyclonal antibody (IgG), Percoll® and RPMI 1640 medium (Sigma-Aldrich Co. St. Louis, MO, USA) were also commercially purchased.

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Porcine PBMCs and PMNs

Clinically healthy 6-month-old crossbred pigs from slaughterhouse (Donga food Co. Ltd., Cheongju, Korea) were used as blood donors. The peripheral blood drawn from anterior vena cava of pigs was immediately filled in heparinized conical tube. To isolate PBMCs, blood was overlaid on a Percoll® solution (specific gravity, 1.080). After centrifugation for 45 min at 400 g at room temperature, PBMCs in the interface between the Percoll® solution and plasma were harvested. To purify the PBMCs, they were pelleted by centrifugation at 900 g for 5 min, washed with phosphate-buffered saline (PBS), treated with 0.83% ammonium chloride in a Tris-base buffer (pH 7.2) for 3 min to destroy remaining erythrocytes. RPMI 1640 medium containing 5% heat-inactivated FBS was used to resuspend PBMCs.

The PMNs were collected from layer of erythrocyte sediment. One ml of upper part of erythrocyte sediment was harvested. Erythrocyte sediment was mixed with 9 ml of 1.5% dextran (mw, 200,000; Wako Ltd., Osaka, Japan) solution and allowed to sediment for 60 min. Remaining erythrocytes were lysed by treatment with 0.83% ammonium chloride solution, washed two times with PBS. Resulting PMNs were suspended in RPMI 1640 medium. Viability of the PMNs and PBMCs was verified to be > 95% by method of trypan blue dye exclusion.

Culture supernatants

PBMCs (2×10^6 cells/ml) treated with zinc sulfate (0-200 μM) were suspended in RPMI 1640 medium supplemented with 5% FBS and trace amount of penicillin/streptomycin to prevent contamination. PBMCs placed in 24-multiwell plates (Nunc Co., Naperville, IL, USA) and incubated at 37°C under 5% CO_2-humidified environment for 24 h. After incubation, culture supernatants were centrifuged at 14,000 × g for 10 min, filtered through membrane filter of 0.2 μm-pore size, and stored at −70°C until used.

Chemotaxis assay

The chemotaxis chambers (Neuro probe, Gaithersburg, MD, USA) were used to determine directional migration distance of PMNs as described previously, (Jeon *et al*., 2012; Watanabe *et al*., 1985). Briefly, chemotaxis chambers, RPMI 1640 medium and culture supernatants from PBMCs were pre-warmed at 37°C incubator for 2 h. Two hundreds μl of zinc solution or culture supernatants from PBMCs treated with zinc was placed in lower chamber of device. A nitrocellulose filter (150 μm thick and 3.0 μm pore size; Merk-Millipore Corporation, Frankfurter, Darmstadt, Germany) was placed on top of the well of the lower compartment. And then, 200 μl of PMNs suspension (4×10^5 cells/200 μl) was put into the upper compartment and the chambers were incubated for 45 min at 37°C under 5% CO_2-humidified atmosphere. After incubation, the membrane filters were immediately removed by pincette, fixed for 20 sec in 70% ethyl alcohol and washed by 100% ethyl alcohol, dried for 1 h and stain with harris-hematoxylin for 4 min, decolorized with 70% ethyl alcohol for 20 sec and dried on kims-wipe paper. The membrane filters were mounted on slide glass with cytoseal XYL (Thermo-Fisher scientific, Waltham, MA, USA).

A bright field microscopy at 400 × magnification was used to determine the directional migration distance of cells. Five fields per filter were selected randomly to examine migration distance of cells in triplicate assay. The chemotactic response of PMNs was assessed as the absolute distance (μm/45 min) in the directional migration of PMNs in response to chemoattractant materials.

Neutralization test

Anti-recombinant porcine IL-8 polyclonal antibody (anti-rpIL-8 pAb) (50 μg/ml) was added to the culture supernatants from PBMCs treated with 200 μM of zinc. As a control isotype IgG, 50 μg/ml of anti-recombinant mouse IL-6 polyclonal antibody (anti-rmIL-6 pAb) instead of anti-rpIL-8 pAb was added to the well. The mixed samples were placed for 2 h at room temperature. The chemotactic activity of PMNs by pAb-treated culture supernatants was also evaluated as described above.

Heat stability test

To test the temperature stability of chemoattractant factor (s), culture supernatants from PBMCs treated with zinc (200 μM) were exposed to temperature 4, 37, 70 and 85°C for 15 min. The chemotactic activity of PMNs by heat-treated culture supernatants was also evaluated as described above.

Statistical analyses

All statistical analyses by using GraphPad Prism 6 for windows (GraphPad Software, Inc., San Diego, CA, USA) were carried out. Results were compared by one-way analysis of variance (ANOVA) followed by Tukey's test or Student's *t*-test. P value of under 0.05 was considered statistically significant. Results were expressed as means ± standard deviation (SD).

Results

Direct effect of zinc on chemotaxis of PMNs

To examine the direct effect of zinc on chemotaxis of PMNs, the migrated distance of PMNs in response to zinc was measured. The direct treatments of zinc at concentrations of 10 to 200 μM showed no effect on chemotaxis of PMNs as compared with controls (Fig 1).

Chemotactic activity of porcine PMNs by culture supernatant from PBMCs treated with zinc

To examine the effect of culture supernatants from zinc-treated PBMCs on chemotaxis of PMNs, the migrated distance of PMNs in response to culture supernatant from PBMCs (2×10^6 cells/ml) treated with zinc (0-200 μM) for 24 h was measured. Chemotactic activity of PMNs by culture supernatant from PBMCs treated without zinc (0 μM) for 24 h was significantly higher ($P < 0.001$) than that of medium alone. Culture supernatants from PBMCs treated with zinc at concentrations of 100-200 μM but not 10-50 μM remarkably increased ($P < 0.001$) the chemotactic activity of PMNs when compared with that without zinc (0 μM) for 24 h (Fig 2). Also, diluted culture supernatants (12.5-100%) from PBMCs treated with 200 μM of zinc for 24 h significantly

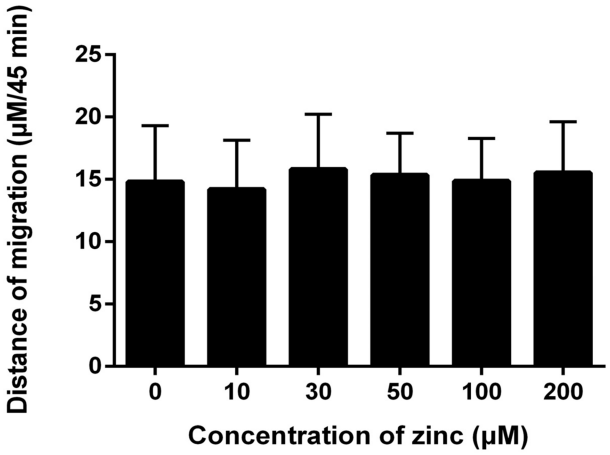

Fig 1. Direct effect of zinc on chemotactic activity of porcine PMNs. Two hundred microliter of freshly isolated PMNs (4×10^5 cells/200 μl) was filled in upper chamber and zinc (0-200 μM) was placed in the lower chamber. Migration distance of PMNs after incubation for 45 min was measured. One-way ANOVA followed by Tukey's test was used to investigate differences between control and zinc-treated groups. Data represent the means ± SD (n = 3).

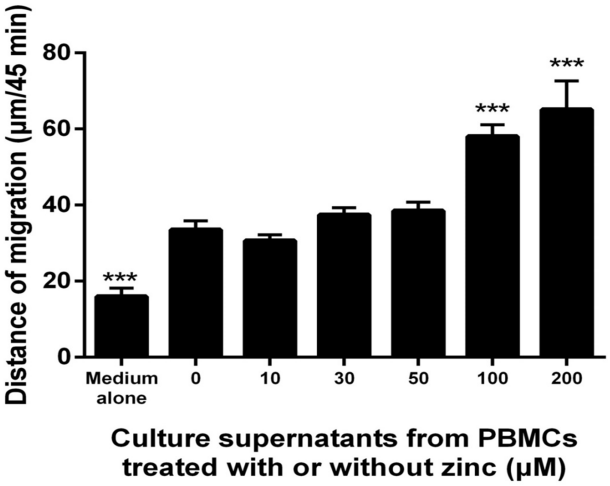

Fig 2. Chemotactic activity of PMNs by culture supernatants from PBMCs exposed to various concentrations (0-200 μM) of zinc. Two hundred microliter of freshly isolated PMNs (4×10^5 cells/200 μl) was filled in upper chamber and 200 μl of culture supernatants from PBMCs (2×10^6 cells/ml) treated with zinc (0-200 μM) for 24 h was placed in the lower chamber. Then, migration distance of PMNs after incubation for 45 min was measured. One-way ANOVA was used to investigate differences between culture supernatants from PBMCs without zinc (0 μM; control) and other treatments, followed by Tukey's test. Data represent the means ± SD (n = 3). ***P < 0.001, compared to control group (0 μM).

increased (P < 0.05-0.001) the migration distance of PMNs in a dose-dependent manner when compared with the medium alone (0%). This chemotactic activity was peaked at 100% of culture supernatant (Fig 3).

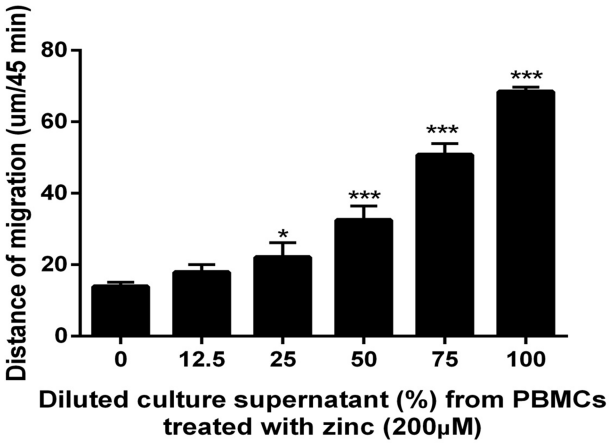

Fig 3. Chemotactic activity of PMNs by diluted culture supernatants from PBMCs exposed to zinc of 200 μM. Two hundred microliter of freshly isolated PMNs (4×10^5 cells/200 μl) was filled in upper chamber and culture supernatants from zinc (200 μM)-treated PBMCs diluted with RPMI 1640 medium were placed in the lower chamber. Then, migration distance of PMNs after incubation for 45 min was measured. One-way ANOVA was used to investigate differences between 0% and treated groups, followed by Tukey's test. Data represent the means ± SD (n = 3). *P < 0.05, ***P < 0.001 vs control (0%).

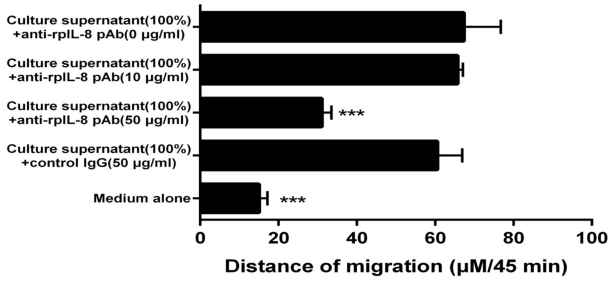

Fig 4. Neutralization effect of anti-rpIL-8 pAb on chemotactic activity of PMNs by culture supernatant (100%) from PBMCs treated with zinc (200 μM). Anti-rpIL-8 pAb (IgG) was mixed to culture supernatant from PBMCs treated with zinc for 2 h. As a control isotype IgG, anti-rmIL-6 pAb (IgG) was used. One-way ANOVA was used to investigate differences between 100% culture supernatant and pAb-treated groups, followed by Tukey's test. Data represent the means ± SD (n = 3). ***P < 0.001 vs 100% culture supernatant.

Neutralization effect of anti-rpIL-8 pAb on chemotactic activity of PMNs by culture supernatant from zinc-treated PBMCs

To examine whether the increased chemotactic activity of PMNs by culture supernatant from PBMCs treated with zinc is due to IL-8, the neutralization effect by anti-rpIL-8 pAb was tested. As shown in Fig 4, the increased chemotactic activity of PMNs by culture supernatant from PBMCs treated with zinc (200 μM) for 24 h was significantly inhibited (P < 0.001) by anti-rpIL-8 pAb at concentration of 50 μg/ml when compared with that of 100% culture supernatant. However, in the examination of the possibility of nonspecific inhibition for

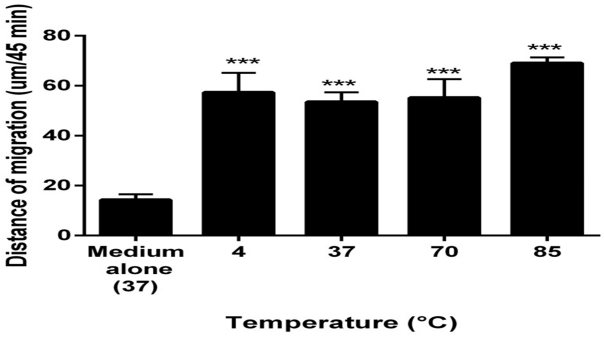

Fig 5. Effect of heat treatment on chemotactic activity of PMNs by culture supernatant (100%) from PBMCs treated with zinc (200 μM). Culture supernatants from PBMCs treated with zinc were treated at 4, 37, 70 and 85°C for 15 min. Student's *t*-test was used to investigate differences between medium alone group and heat-treated culture supernatant groups. Data represent the means ± SD (n = 3). ***P < 0.001, compared to medium alone.

immunoglobulin isotype, IgG, chemotactic activity of PMNs was not inhibited by control IgG, anti-rmIL-6 pAb (50 μg/ml) instead of anti-rpIL-8 pAb (Fig 4).

Effect of heat treatment on chemotactic activity of PMNs by culture supernatant from PBMCs treated with zinc

To investigate the physical characteristics of culture supernatant from PBMCs treated with zinc, heat stability was tested. The chemotactic activity by culture supernatant from zinc-treated PBMCs was unaffected by temperature from 4 to 85°C for 15 min (Fig 5).

Discussion

It was reported in the previous study (17) for influence of zinc on cell viability that zinc at concentration around 250 μM decreased cell viabilities of PMNs and PBMCs when incubated for 24 hours. Thus, zinc in this study was used at concentrations of < 200 μM showing no cytotoxic effect and high cell viability. Although it was revealed that zinc (> 500 μM) can directly stimulate migration of PMNs through activation of polarization reaction (11), zinc of 0 to 200 μM we tested did not show any direct migration effect on PMNs. It was conceivable that < 200 μM of zinc may be directly less responsible for PMNs chemotaxis.

Our results showed that culture supernatant from PBMCs without zinc increased the chemotactic activity of PMNs compared to medium alone. It was thought that even if PBMCs were treated without zinc for 24 hours, they can secrete trace amount of soluble chemoattractant products (21,23).

We found that culture supernatants from PBMCs exposed to zinc (100-200 μM) increase the chemotactic activity of PMNs. It is corresponded with previous study that > 100 μM of zinc dosage can activate monocytes/macrophage directly (12). It could be assumed that zinc stimulates monocytes and/or lymphocytes to produce chemoattractant factor (s). This strongly suggests that PBMCs treated with zinc (100-200 μM) may be activated to produce chemotactic cytokines such as IL-1β and IL-8. Although IL-1β has chemotactic activity of PMNs through stimulation of endothelial cells to produce a neutrophil chemotactic factor *in vivo*, it does not show chemotactic activity of PMNs *in vitro* (26). It was known that recombinant or highly purified IL-1 does not affect chemotactic activity of PMNs (25). IL-8 with molecular weight 6 to 8 kDa is most important chemoattractant factor responsible for chemotactic activity of PMNs (3). Therefore, we hypothesized that zinc stimulate PBMCs to produce IL-8 like factor, which increases the chemotaxis of PMNs. To prove this hypothesis, we examined the neutralization effect of anti-rpIL-8 pAb on chemotactic activity of PMNs. The anti-rpIL-8 pAb negated the chemotactic activity of PMNs increased by culture supernatant from PBMCs treated with zinc. We also tested the nonspecific neutralization effect of antibody isotype, IgG, using anti-rmIL-6 pAb. However, the anti-rmIL-6 pAb did not neutralize the chemotactic activity of PMNs. These findings suggest that the chemotactic factor from PBMCs stimulated by zinc is IL-8. We also tested heat stability of soluble product (s) in culture supernatant. The soluble product (s) were stable in heated (4-85°C) condition. This corresponded with physical characteristics of IL-8 (2,22). Therefore, these data strongly indicated that IL-8 exists in culture supernatant from zinc-treated PBMCs.

IL-8 is basic protein contains two disulfide *bridges* formed by four cysteins. Main targets of IL-8 are neutrophils. It has effects on neutrophils such as shape change and directional migration, exocytosis of storage protein, respiratory burst and leak out from vessel wall to site of inflammation (2,9).

A previous study (17) founded that zinc not only directly stimulate phagocytic capacity of monocyte but also stimulate PBMCs to produce TNF-α to enhance phagocytic capacity of PMNs and monocytes. Our data also indicate that zinc can stimulate PBMCs to produce IL-8 chemotactic factor. Therefore, it can be thought that zinc upregulate the capacity of phagocytic cells including monocytes and neutrophils mediated through TNF-α and the chemotactic activity of neutrophils through IL-8, which are produced by PBMCs. These findings strongly suggest that zinc has immunostimulating effects on phagocytosis of phagocytic cells and chemotaxis of PMNs.

In veterinary medicine, oral supplement of zinc with drugs in cutaneous fungal infection of beef cattle exhibited synergism effect (8). Zinc-supplementation to piglet reduced subclinical inflammation in the ileo-ceco-colic areas (14). Dietary zinc supplementation to weaned pigs can decreased bacterial translocation from the small intestine to the ileal mesenteric lymph node (10). Zinc supplementation can potentially improve the both cellular and humoral immune response (20). Therefore, the co-administration of zinc will be able to increase the host defense ability of animals with diseases including post weaning diarrhea, skin fungal infection, and immune-illness.

In conclusion, our results suggested that zinc enhances the chemotactic activity of porcine PMNs through the increased IL-8 chemotactic factor from zinc-treated PBMCs.

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