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ORIGINAL ARTICLE

Zinc increases the phagocytic capacity of canine peripheral blood phagocytes *in vitro*

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Abstract Zinc is a trace element that plays a central role in the immune system. In the present study, the effect of zinc on the phagocytic capacity of canine peripheral blood phagocytes was examined *in vitro* by flow cytometry. Zinc was used at a concentration of 100 μM, which preserved cell viability. Treatment with zinc did not directly affect the phagocytic capacity of peripheral blood polymorphonuclear neutrophils (PMN) and mononuclear cells (PBMC). However, it did directly enhance the phagocytic capacity of peripheral blood monocyte-rich cells. Moreover, the phagocytic capacity of PMN and monocyte-rich cells but not PBMC was remarkably enhanced by culture supernatants from PBMC but not PMN treated with zinc. Anti-recombinant canine (rc) tumor necrosis factor-alpha (TNF-α) polyclonal antibody (pAb) neutralized the enhancing effect of the culture supernatant from zinc-treated PBMC and this supernatant had higher TNF-α levels than the culture supernatant of untreated PBMC. Thus, zinc may stimulate canine PBMC to produce TNF-α, which enhances the phagocytic capacity of canine peripheral blood phagocytes.

Keywords Canine · Neutrophils · Phagocytosis · Tumor necrosis factor-alpha · Zinc

Abbreviations

FITC	fluorescein isothiocyanate
IL	interleukin
pAb	polyclonal antibody
PBMC	peripheral blood mononuclear cells
PMN	peripheral blood polymorphonuclear neutrophils
rm	recombinant mouse
rc	recombinant canine
TNF-α	tumor necrosis factor-alpha

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Introduction

Neutrophils and monocytes are the first line of defense against bacterial infections (Underhill and Ozinsky 2002) and act to defend the host by chemotaxis, phagocytosis, releasing lysosomal enzymes and generating reactive oxygen metabolites (Eickhoff et al. 2004; Underhill and Ozinsky 2002). The first step in this process is the recognition of and attachment to the target. These responses are modulated by cytokines, including interleukin (IL)-1, IL-2, IL-8, interferons and tumor necrosis factors, that are released by peripheral blood mononuclear cells (PBMC) (Boyaka and McGhee 2001; Trinchieri 1995; Zhao et al. 1997).

Since zinc is an essential nutrient that is required by mammals for many physiological functions (Vallee and Falchuk 1993), it has been suggested that impaired immune functions can be revitalized by pharmacological supplementation of zinc (Rink and Gabriel 2000). Moreover, zinc-responsive dermatosis (White et al. 2001) and hepatic copper toxicosis (Brewer et al. 1992) in human and veterinary medicine can be treated with zinc. It has been suggested that zinc deficiency may disturb the functions of innate immunity and that this increases susceptibility to a variety of pathogens (Keen and Gershwin 1990; Prasad 2007; Rink and Gabriel 2000; Rink and Kirchner 2000). Supporting this are *in vivo* studies showing that low zinc levels can impair macrophage and neutrophil phagocytosis, oxidative burst generation, and natural killer cell activity, and that granulocyte numbers decrease during zinc deficiency (Allen et al. 1983; Keen and Gershwin 1990). In addition, it was reported recently that zinc deficiency reduces T-cell IL-2 production in humans and rats (Beck et al. 1997; Hosea Blewett et al. 2008) and reduces the interferon-γ production by human T lymphoblast cell lines 6 h after 12-myristate 13-acetate (PMA)/*p*-phytohemagglutinin (PHA) stimulation (Bao et al. 2003). Zinc restriction also increases the production of IL-1β by human PBMC cultured in serum-free medium (Wellinghausen et al. 1996). However, the production of IL-4, IL-6, or IL-10 by PBMC is not affected by the zinc status (Bao et al. 2003). It is noteworthy that these zinc deficiency-induced changes can be reversed by zinc supplementation (Beck et al. 1997). Moreover, zinc directly induces chemotactic activity in human polymorphonuclear leukocytes (Chavakis et al. 1999; Hujanen et al. 1995). However, the optimal therapeutic dosage that is required to reverse the immunological effects of zinc deficiency is still unclear (Ibs and Rink 2003). It is important that this zinc dose is close to the actual requirement as too much zinc can result in zinc toxicity and negative side effects on immune functions (Ibs and Rink 2003; Mikszewski et al. 2003).

To our knowledge, little is known regarding the effect of zinc on canine peripheral blood leukocyte immune responses. Thus, we sought to examine the effect of *in vitro* treatment with zinc sulfate on the phagocytic capacity of canine peripheral blood phagocytes. We also examined the effect of zinc treatment on tumor necrosis factor (TNF)-α production by canine PBMC.

Materials and methods

Animals

Clinically healthy Beagle dogs were used as blood donors. All dogs were housed at room temperature (22±2°C) with a light cycle of 12 h day and 12 h night. All dogs were individually managed in cages and fed on a pellet diet (ProPlan; Purina Korea, Seoul, Korea). All experimental procedures and the use of animals were approved by the ethics committee of the Chungbuk National University.

Reagents

The zinc sulfate solution (Fluka Chemie AG, Buchs, Switzerland) was purchased and sterilized by being passed through a 0.45-μm-membrane filter (Milipore Co., Bedford, Mass, USA) before being used as a stock solution of 50 mM. Goat anti- recombinant canine (rc) TNF-α polyclonal antibody (pAb) (IgG) (R&D Systems Inc., Minneapolis, MN, USA) and rabbit anti-recombinant mouse (rm) IL-6 pAb (IgG) (Sigma-Aldrich Co., St. Louis, MO, USA) were also purchased.

Canine PBMC and PMN isolation

Peripheral blood drawn in a heparinized tube from the jugular vein was layered on an equal volume of Percoll™ solution (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and centrifuged at 400×g for 40 min at room temperature. The resulting PBMC layer at the interface between the plasma and Percoll solution was harvested and treated with 0.83% NH_4Cl Tris-base buffer (pH 7.2) for 5 min to lyse the remaining erythrocytes. The PBMC layer consisted of approximately 30% monocytes and 70% lymphocytes, as determined by cell counting after Wright-Giemsa staining. The PMN were obtained from the centrifuged gradient layer of the erythrocyte sediment after collecting the PBMC. One milliliter taken from the upper portion of the erythrocyte layer was mixed with 10 ml of 1.5% dextran (molecular weight, 200,000; Wako Ltd., Osaka, Japan) in PBS and allowed to sediment for 45 min. The residual erythrocytes were lysed by treatment with 0.83% NH_4Cl solution for 5 min at 37°C, after which the cells were washed three times with PBS. The purity of the neutrophils in the final canine PMN suspension was routinely greater than 96%, as determined by cytospin smear and Wright-Giemsa staining analyses. The viability of the PBMC and PMN, as determined by trypan blue dye exclusion, exceeded 97% in all cases. All cells were resuspended in RPMI 1640 medium (Sigma-Aldrich Co.) supplemented with 2 mM L-glutamine, 0.02 mg/ml gentamicin and 5% heat-inactivated fetal bovine serum (Gibco Co., Grand Island, NY, USA). The cell concentration was finally adjusted to 2×10^6 cells/ml for making culture supernatant or to 1×10^6 cells/ml for evaluating phagocytic capacity.

Viability assay

Canine PBMC and PMN (2×10^6 cells/ml) were incubated with or without different concentrations of zinc sulfate in 24-well plastic culture plates for 24 h at 37°C, and cell viability was determined by determining trypan blue exclusion.

Culture supernatant generation

The isolated PBMC or PMN at a density of 2×10^6 cells/ml were placed in wells of a 24-multiwell plate (Nunc Co., Naperville, IL, USA) and incubated with or without 100 μM of zinc sulfate for 24 h at 37°C in a 5% CO_2-humidified atmosphere. After 24 h incubation, the culture supernatants were collected, centrifuged at 5,000×g for 30 min, filtered through a 0.45-μm pore size membrane filter and stored at −70°C until use.

Phagocytic capacity analysis

PMN or PBMC adjusted to 1×10^6 cells/ml were incubated for 12 h at 37°C in a 5% CO_2-humidified atmosphere with zinc sulfate-containing media or with the culture supernatant

from zinc sulfate-treated (100 μM) PBMC. Twenty microliter of 1×10^9 beads/ml fluorescein isothiocyanate (FITC)-latex beads (size, 2.0 μm; Sigma-Aldrich Co.) was added to each well for the final 1 h of incubation. PMN or PBMC incubated without FITC-latex beads served as negative controls. The cultured cells were harvested gently by slow pipetting, centrifuged at 400×g for 3 min, and washed three times with PBS containing 3 mM ethylenediamine tetraacetic acid (EDTA). The phagocytic capacity of the phagocytes was analyzed by flow cytometry (FACS Calibur; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) using CELLQuest software. The argon laser was set to emit an excitation wavelength of 488 nm. The FITC (green) fluorescence of 10,000 cells per sample was measured between 515 and 560 nm. The cells were gated on the basis of forward and side light scatter characteristics. For analysis, the monocyte-rich cell population was identified by its typical location on the PBMC scattergram and was selected by gating. The results were expressed as percentages of total phagocytic capacity.

Neutralization test

Various concentrations of anti-rcTNF-α (IgG) were added to the zinc sulfate-treated PBMC culture supernatant. Rabbit anti-rm IL-6 pAb served as a control isotype IgG. The samples were maintained at room temperature for 30 min. The effect of these samples on the phagocytic capacity of the PMN and monocyte-rich cell populations was evaluated as described above.

Measurement of TNF-α in the culture supernatant of zinc-treated PBMC

The TNF-α levels in the culture supernatants of PBMC treated with or without zinc sulfate (100 μM) were determined by the direct sandwich enzyme-linked immunosorbent assay (ELISA) in the Quantikine® TNF-α immunoassay kit (R&D System Inc.). The optical density was determined by using an automated microplate reader (ELx808, Bio-Tek Instruments Inc., Winooski, Vermont, USA) at 450 nm. The TNF-α levels in the samples were quantified from standard curves generated with purified canine TNF-α tested at eight titration points. The lower and upper detection limits were 7.8 and 500 pg/ml, respectively.

Statistical analysis

All statistical analyses were carried out by using SigmaStat version 2.03 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to investigate differences between the control and concentrations of zinc sulfate treatment, followed by Dunnett's *post hoc* test. Comparison of two groups was made by *t* test. $p<0.05$ was considered statistically significant. All data were expressed as mean values and standard deviation (SD).

Results

Effect of zinc sulfate on canine PBMC and PMN cell viability

PBMC and PMN preparations with > 97% viability were cultured for 24 h with zinc sulfate at concentrations ranging from 0 to 250 μM, after which their viability was assessed by determining trypan blue dye exclusion. Zinc sulfate at the higher concentration of 250 μM

Table 1 Cell viability (%) of PBMC and PMN treated with zinc sulfate for 24 hours

Cell type	Zinc sulfate concentration (μM)				
	0	25	50	100	250
PBMC (%)	96.33±1.53	97.00±1.00	96.00±1.00	94.00±1.00	92.67±0.58*
PMN (%)	98.67±0.58	99.00±1.00	98.00±1.73	97.00±1.73	94.67±0.58*

The values represent mean ± SD (n=5). * $p<0.05$ *versus* control (0 μM zinc sulfate)

lowered the viability of the PBMC ($p=0.002$) and PMN ($p=0.010$) as compared to the viability of the untreated control cells (0 μM zinc sulfate) (Table 1). Thus, zinc sulfate was used in subsequent experiments at a concentration of 100 μM, which does not appear to affect cell viability.

Direct effect of zinc sulfate on the phagocytic capacity of peripheral blood leukocytes

To examine the direct effect of zinc sulfate on the phagocytic capacity of peripheral blood leukocytes, freshly isolated PMN and PBMC were cultured for 12 h with zinc sulfate at concentrations ranging from 0 to 100 μM. The phagocytic capacity of PMN ($p=0.446$) or PBMC ($p=0.789$) was not enhanced by zinc (Table 2). However, the phagocytic capacity of the monocyte-rich cells, which were identified by their typical location on the PBMC scattergram during flow cytometric analysis, was enhanced by 50 and 100 μM zinc ($p=0.009$) (Table 2).

Effect of the culture supernatant of zinc sulfate-treated PMN on the phagocytic capacity of peripheral blood leukocytes

We also examined the effect of treating the phagocytes with the culture supernatants from zinc-treated PMN. As shown in Table 3, this culture supernatant did not enhance the phagocytic capacity of the PMN ($p=0.526$), PBMC ($p=0.795$), or monocyte-rich cells ($p=0.741$).

Effect of the culture supernatant of zinc sulfate-treated PBMC on the phagocytic capacity of canine peripheral blood phagocytes

To examine whether the phagocytic capacity of peripheral blood leukocytes can be enhanced by the culture supernatant from zinc sulfate-treated PBMC, freshly prepared PMN and PBMC were incubated for 12 h with culture supernatant (0 to 100%) from PBMC

Table 2 Direct effect of zinc sulfate on the phagocytic capacity (%) of PMN, PBMC, and the monocyte-rich cell population

Cell type	Zinc sulfate concentration (μM)			
	0	25	50	100
PMN (%)	2.17±0.40	1.94±0.61	1.64±0.29	1.59±0.54
PBMC (%)	10.15±1.94	10.41±0.88	11.38±1.96	10.54±1.07
Monocyte-rich cell population (%)	28.33±1.89	28.80±1.84	34.34±3.12*	36.31±2.78*

The values represent mean ± SD (n=4). * $p<0.05$ *versus* control (0 μM zinc sulfate)

Table 3 Effect of zinc sulfate-treated PMN culture supernatant on the phagocytic capacity (%) of PMN, PBMC, and the monocyte-rich cell population

Cell type	Zinc sulfate-treated PMN culture supernatant (%)					
	0	12.5	25	50	75	100
PMN (%)	2.14±0.77	2.09±0.65	1.86±0.42	2.00±0.37	2.75±0.74	2.44±0.57
PBMC (%)	14.36±0.70	13.87±0.73	14.37±1.31	13.60±1.66	13.08±1.91	13.27±1.49
Monocyte-rich cell population (%)	22.40±2.52	23.14±2.68	24.09±1.85	23.88±1.43	22.98±2.22	21.48±2.64

No significant differences between the control (0%) and treatments were noted. The values represent mean ± SD (n=4)

(2×10^6 cells/ml) treated with 100 μM zinc sulfate for 24 h. The phagocytic capacity of the PMN was remarkably enhanced in a dose-dependent manner by the culture supernatant (p=<0.001; Fig. 1a). However, the culture supernatant had no effect on the phagocytic capacity of PBMC (p=0.849) (Fig. 1b). The phagocytic capacity of the monocyte-rich cells was also enhanced by 12.5%, 25%, and 50% of the culture supernatant (p=<0.001; Fig. 1c).

Ability of anti-rcTNF-α pAb to neutralize the phagocytosis-enhancing ability of the zinc-treated PBMC culture supernatant

To confirm that TNF-α is the factor in the culture supernatant that is responsible for its phagocytosis-enhancing effects, we examined whether an anti-rcTNF-α pAb would neutralize these culture supernatant-induced effects. Indeed, when used at concentrations of 0.001 to 1 μg/ml, anti-rcTNF-α pAb inhibited, in a dose-dependent manner, the enhanced phagocytic capacities of PMN (p=<0.001; Fig. 2a) and monocyte-rich cells (p=<0.001; Fig. 2b) induced by 100% of the culture supernatant. To ensure that this inhibitory effect was specific, we also tested the effect of a high concentration (1 μg/ml) of anti-rmIL-6 pAb, which served as an immunoglobulin IgG isotype control. No nonspecific inhibition was observed (Fig. 2a and b).

Amount of TNF-α in the culture supernatant from zinc sulfate-treated PBMC

The amount of canine TNF-α in the culture supernatant (100%) from PBMC treated with zinc sulfate (100 μM) for 24 h was quantified. The level of TNF-α in zinc sulfate-treated PBMC culture supernatant was significantly higher than the level in the supernatant from PBMC cultured without zinc (p=<0.001) (Fig. 3).

Discussion

In the present study, we showed that while zinc sulfate at concentrations around 250 μM affected the viability of canine PBMC and PMN composing of mostly neutrophils, zinc at 100 μM had no apparent effect on cell viability. In contrast, a previous study showed that zinc at concentrations of 100 μM or higher decreased the viability of human PBMC (Chang et al. 2006). We also showed that while treatment with 100 μM zinc for 12 h did not directly affect the phagocytic capacity of canine PBMC, it did directly enhance the

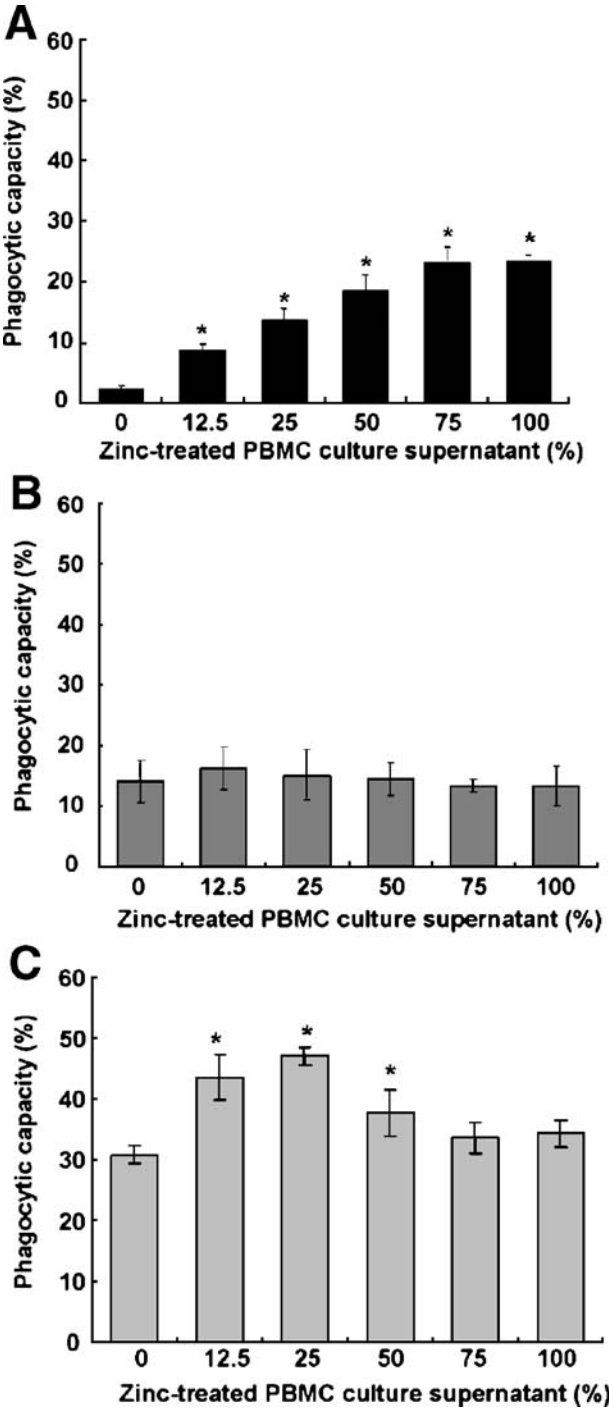

Fig. 1 Effect of zinc-treated PBMC culture supernatant on the phagocytic capacity of PMN (**a**), PBMC (**b**), and the monocyte-rich cell population (**c**). Freshly isolated cells (1×10^6 cells/ml) were incubated for 12 h with 0–100% culture supernatant from PBMC (2×10^6 cells/ml) that had been treated with zinc sulfate (100 μM) for 24 h. FITC-latex beads were added to the cultures for the final 1 h. The phagocytic capacity was measured by using flow cytometry, and one-way ANOVA was employed to test differences between the control and treatments, followed by Dunnett's *post-hoc* test. The values indicate means ± SD (n=4). *$p<0.05$, compared with 0% culture supernatant (100% medium)

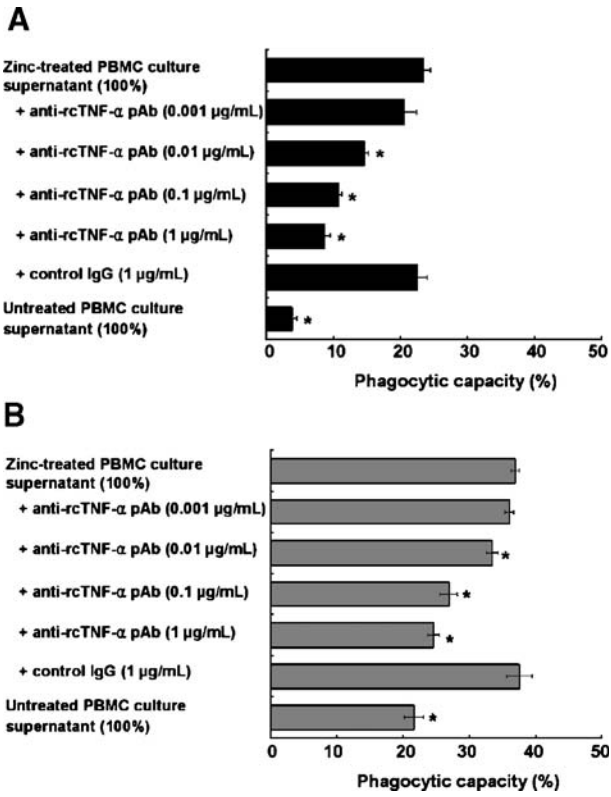

Fig. 2 Neutralizing effect of anti-rcTNF-α pAb on the phagocytic capacity of PMN (**a**) and the monocyte-rich cell population (**b**). Anti-rcTNF-α pAb was added at the indicated concentrations to the culture supernatant of PBMC treated with or without zinc sulfate (100 μM) for 24 h. Goat anti-rmIL-6 pAb served as a control isotype IgG. The antibody-supernatant mixtures were left for 30 min before being added to the cells. After incubation for 12 h, FITC-latex beads were added for the final 1 h. The phagocytic capacity was measured by flow cytometry, and one-way ANOVA was used to investigate differences between the control and treatments, followed by Dunnett's *post hoc* test. The values are means ± SD (n= 4). *$p<0.05$, compared with zinc-treated PBMC culture supernatant (100% zinc-treated supernatant)

phagocytic capacity of canine monocytes. This probably reflects the fact that the monocytes in the canine PBMC comprise just 30% of all the cells, with the remainder being lymphocytes. In addition, we found that zinc treatment had no direct effect on canine PMN. This may reflect the fact that PMN phagocytosis peaks within 5 h after phagocytosis is stimulated and then wanes (Kang and Yang 2008; van Eeden et al. 1999). The beneficial effect of zinc on immune system has been widely documented. However, the direct effects

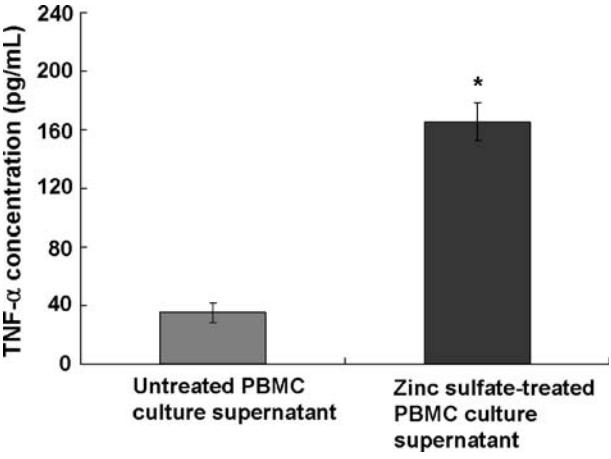

Fig. 3 TNF-α production in canine PBMC by zinc sulfate treatment. The amount of TNF-α in the culture supernatant from PBMC (2×10^6cells/ml) treated with or without zinc sulfate (100 μM) for 24 h was determined using an ELISA. Comparison of two groups was made by *t*-test. The values are means ± SD (n=4). *$p<0.05$

on cell functions are controversial because an inhibitory effect of zinc on immune cells has been also reported (Chvapil et al. 1977; Vega-Robledo et al. 2007). Moreover, recent reports have warranted even that zinc supplementation may result in detrimental influences to host. It has been reported that treatment with zinc can have an inhibitory effect on the viability and differentiation of human monocytes and murine bone marrow macrophages (Vega-Robledo et al. 2007), and that excessive intake of zinc may trigger suicidal death of circulating erythrocytes, leading to development of anemia (Kiedaisch et al. 2008).

Notably, we observed that the phagocytic capacities of canine PMN and monocytes were enhanced by the culture supernatant from zinc sulfate-stimulated PBMC. This suggests that there is a soluble factor(s) in zinc-treated PBMC culture supernatant that up-regulates the phagocytic capacity of phagocytes. One such factor may be TNF-α (Kang et al. 2007; Shalaby et al. 1985). Indeed, we found that zinc sulfate treatment increased the TNF-α production of canine PBMC. Anti-rcTNF-α pAb completely neutralized the ability of the culture supernatant from zinc-treated PBMC to enhance the phagocytic capacity of canine PMN and monocytes. Thus, zinc stimulates canine PBMC to produce TNF-α, which in turn enhances the phagocytic capacity of phagocytes. Several reports support these findings. One study showed that TNF-α can increase neutrophil phagocytosis and superoxide generation (Mullen et al. 1995), while others have found that zinc increases the TNF-α production of human PBMC (Driessen et al. 1994; Salas and Kirchner 1987; Scuderi 1990) and mouse mast cells (Yamasaki et al. 2007). It has been also shown that the release of TNF-α after PBMC stimulation with zinc involves *de novo* transcription of mRNA rather than enhanced translation of already-expressed mRNA (Wellinghausen et al. 1996). Zinc may stimulate the production of TNF-α by activating intracellular protein tyrosine and cAMP-and cGMP-dependent protein kinase (Wellinghausen et al. 1996) and/or by functioning itself as a novel intracellular second messenger (Yamasaki et al. 2007). Recently, it has been emphasized that TNF-α plays a critical role in host defense (Bongartz et al. 2006; Chen et al. 2000), although the excessive synthesis and release of TNF-α can oppositely have in a variety of harmful effects. Thus, our observation that zinc could induce TNF-α production from canine PBMC may indicate the possible utility of zinc *in vivo*.

It is possible that the monocytes in the PBMC population are responsible for the zinc-induced TNF-α production of PBMC since only the monocyte-rich population of the PBMC showed phagocytosis enhancement upon zinc treatment. Several studies have also shown that the secretion of TNF-α is induced in monocytes directly and is not dependent on the presence of lymphocytes (Rühl and Kirchner 1978; Wellinghausen et al. 1997). Thus, zinc may stimulate monocytes to produce TNF-α, which then acts in an autocrine manner to promote monocyte phagocytosis. Alternatively, zinc may act directly to promote monocyte phagocytosis without having to evoke monocyte TNF-α secretion first. It appears that PMN do not secrete TNF-α upon zinc treatment, as the culture supernatant from zinc-treated PMN had no effect on phagocyte function. Instead, it seems that zinc stimulates PMN only indirectly.

In summary, *in vitro* treatment of PBMC with zinc could induce TNF-α production, which increases the phagocytic capacity of canine peripheral blood phagocytes such as PMN and monocyte-rich cells.

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