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Effect of Methylprednisolone Sodium Succinate on Innate Immune Function of Canine Peripheral Blood Phagocytes

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Abstract : Glucocorticoids (GCs) are the most widely used immunosuppressive agents, but animals treated with GCs may experience deleterious side effects which limit their use in many clinical conditions. In the present study, we examined whether methylprednisolone sodium succinate (MPSS), a glucocorticoid, modulates circulating leukocyte numbers, phagocytic capacity and oxidative burst activity (OBA) of canine peripheral blood phagocytes, and whether tumor necrosis factor-alpha (TNF-α) release is affected by MPSS injection. Neutrophilia and monocytosis were induced by the administration of a high dose of MPSS, which is the recommended protocol for canine patients with acute spinal cord injury. The injection of MPSS decreased the phagocytic capacity of canine PMNs but not PBMCs, and recovered 12 hours (hr) after the completion of MPSS dosing. The OBA of both PMNs and PBMCs was suppressed by MPSS, and restored 24 hr after the completion of dosing. The lipopolysaccharide-induced TNF-α release by PBMCs but not PMNs exposed to MPSS was reduced 12 hr after the completion of dosing, and recovered 48 hr after the completion of dosing. These results suggest that the application of MPSS protocol inhibits the innate immune functions of canine peripheral blood phagocytes for short time relatively.

Key words **:** Canine, Methylprednisolone sodium succinate, Phagocytic capacity, Oxidative burst activity, Tumor necrosis factor-α.

Introduction

Phagocytosis of innate immune cells such as neutrophils and monocytes is central in the elimination of most extracelluar pathogenic foreign microbe (23). Phagocytosis is accompanied by diverse cellular processes such as engulfment, activation of microbial killing mechanisms, and production of cytokines (1,30). When phagocytes are activated by a variety of foreign particles, they are highly effective generating reactive oxygen species (ROS) by a process known as oxidative burst, following activation of a membrane associated nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase (2). ROS derived from superoxide, together with proteases liberated from the granules, are used to kill ingested microbes. During infections, phagocytes represent a substantial source of cytokines such as tumor necrosis factor (TNF)-α (4). Both neutrophils and monocytes have the ability to either express TNF-α mRNA or secrete the related protein *in vitro* in response to lipopolysaccharide (LPS) (12). Those functions of phagocytes represent an important part of the host defense system against invading microorganisms.

Glucocorticoids (GCs) are the most widely used anti-inflammatory and/or immunosuppressive agents (26). However, animals treated with GCs may experience deleterious side effects which limit their use in many clinical conditions. GCs have an inhibitory effect on macrophage production of reactive oxygen molecules in rats (18,33). *In vivo* treatment with a single dose of cortisol or dexamethasone has been shown to cause reduced superoxide anion radical production in peripheral blood leukocytes (31). However, the contradictory results have been also reported. High doses of cortisone *in vivo* did not affect the phagocytic capacity and OBA of rabbit neutrophils (11). It has reported that increased neutrophil ingestion and killing capacity was induced by 6-methylprednisolone (28). A stimulatory effect of GCs on chemiluminescence of human monocytes has been also demonstrated (14).

Methylprednisolone sodium succinate (MPSS) is a glucocorticoid that has free radical-scavenging properties at very high dosages (7,15). In human medicine, it has been suggested that high dose treatment with MPSS is the only neuroprotective regime indicated by the results of the National Acute Spinal Cord Injury Studies (NASCIS) (5). Experimental evidence in cats with spinal cord injury (SCI) suggests that MPSS is useful in limiting the damaging effects of SCI (6). However, the use of MPSS in SCI remains controversial because of insufficient evidence to support the efficacy of MPSS in limiting SCI and the occurrence of immunosuppressive complications in healthy dogs that receive MPSS (20). Moreover, in the NASCIS I I, humans who received MPSS had a 2.6-fold higher incidence of pneumonia (13).

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The NASCIS I I I patients who received MPSS for 48 hours had a two-fold higher incidence of severe sepsis (19). Despite these side effects, the application of a high dose of MPSS has been prescribed for treatment of dogs with SCI.

In the present study, we examined whether MPSS modulates the phagocytic capacity, OBA and circulating number of canine peripheral blood phagocytes. In addition, we assessed whether LPS-induced TNF-α release from peripheral blood leukocytes is affected by MPSS injection.

Materials and Methods

Dogs

The subjects of this study were twelve 3-year-old healthy Beagle dogs. All dogs were kept in individual cages with a 12-hour light/dark cycle, and fed a commercial diet (ProPlan, Nestle Purina PetCare Korea Ltd, Seoul, Korea) and tap water. All experimental procedures and animal use were approved by the ethics committee of the Chungbuk National University.

Experimental protocol

The dogs were divided into two groups : dogs (n = 6) that received normal saline (control group) and dogs (n = 6) that received MPSS (SOLU-MEDROL INJ®, Pfizer Pharmaceuticals Korea, Seoul, Korea). The design of the experimental protocol involved the application of a high dose of MPSS, which is the recommended protocol for patients with acute SCI (39). The MPSS protocol started with an initial intravenous (IV) dose of 30 mg/kg through a peripheral over-the needle catheter, followed 2 hours (hr) later with a dose of 15 mg/kg. Doses of 10 mg/kg of MPSS were then successively injected at 8, 14, 20, and 26 hr. The total injected dose was 85 mg/kg over 26 hr. In the control group, an equivalent volume of normal saline was injected over the same time period.

Determination of peripheral blood leukocyte numbers

Blood was collected by jugular venipuncture before dosing (0 hr) and at 28, 38, 50, and 74 hr after initial dosing (representing 2, 12, 24, and 48 hr after the end of the injection protocol, respectively). Peripheral blood drawn in K_2-EDTA bottle from jugular vein was analyzed by an automated hematology analyzer, Cell-DYN 3500R (Abbott Diagnostics Division, Abbott Laboratories, Abbott Park, IL, USA) and the following variables were measured: total leukocyte, neutrophil, lymphocyte, monocyte, eosinophil, and basophil numbers.

Peripheral blood cells isolation

Blood samples were collected by jugular vein before dosing (0 hr) and at 2, 12, and 24 hr after intravenous injection of MPSS or normal saline. Peripheral blood drawn in heparinized tube from jugular vein was layered on the equal of Percoll solution (GE Healthcare Bio-Science AB, Uppsala, Switzerland) of which specific gravity was adjusted to 1.077 and centrifuged at 400 × g for 45 minutes (min) at room temperature. The resulting PBMCs in interface between plasma and Percoll solution layer was harvested, and treated with 0.83 % NH_4Cl in Tris-base buffer (pH 7.2) for 5 min to lyse remaining erythrocytes. The PMNs was obtained from layer of erythrocyte sediment after PBMCs removal. One milliliter of the upper part of the erythrocytes was mixed with 10 mL of 1.5 % dextran (molecular weight, 200,000; Wako Ltd., Osaka, Japan) in phosphate-buffer saline (PBS) and allowed to sediment for 45 min. The residual erythrocytes were lysed by treatment with 0.83 % NH_4Cl solution in Tris-base buffer (pH 7.2) for 5 min at room temperature and washed 3 times with PBS. All cells were resuspended in RPMI 1640 medium (Sigma-Aldrich Co, St. Louis, MO, USA) supplemented with 2 mM L- glutamine, 0.02 mg/mL of gentamicin and 5 % heat-inactivated fetal bovine serum (Gibco Co, grand Island, NY, USA) and finally adjusted to 1×10^6 cells/mL.

Simultaneous evaluation of the phagocytic capacity and OBA

The phagocytic capacity and OBA were evaluated simultaneously as described previously (20). Briefly, freshly isolated PMNs and PBMCs were incubated for total 2 hr at 37 °C under 5% CO_2-humidified atmosphere. Twenty μL of carboxylate-modified polysterene fluorescent microsphere (size, 1.0 μm) (TransFluospheres, Molecular Probes Inc, Eugene, OR, USA) suspension were adjusted to 1×10^9 beads/mL and then added to each well containing PMNs for the final 1 hr of culture. One μM dihydrorhodamine 123 (DHR-123) (Sigma-Aldrich Co) was added for the final 15 min of culture. Conversion of nonfluorescent DHR-123 into ifluorescent rhodamine 123 by reactive oxygen species was used as a measure of OBA (32). Cultured cells were gently harvested, centrifuged at 400 × g for 3 min at 4 °C and then washed three times with PBS containing 3 mM ethylenediamine tetra-acetic acid (EDTA). Cells were then resuspended in fixation buffer (BD Cytofix, BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. All steps were carried out in the dark. Cells were then analyzed by a flow cytometry, using a FACS Calibur system (Becton Dickinson Immunocytometry Systems, San Jose, CA) and CELLQuest software (Becton Cickinson Immunocytometry Systems) with an argon laser set to an emission wavelength of 488 nm. Samples (10,000 cells per sample) were assayed in triplicate. The FL1 channel was set to 505-545 nm to capture green fluorescent rhodamine-123, and the FL3 channel was set to 630-660 nm to capture the red fluorescent microspheres. The cells based on forward and side light scattering characteristics were gated. The phagocytic capacity and OBA were expressed as percentages and mean fluorescent intensities (arbitrary units), respectively.

Measurement of TNF–α concentration

Blood samples were collected by jugular vein before dos-

ing (0 hr) and at 2, 12, 24, and 48 hr after intravenous injection of MPSS or normal saline. The isolated PMNs and PBMCs at density of 2×10^6 cells/mL in a well of a 24-multiwell plate (Nunc Co, Naperville, IL, USA) were respectively incubated for total 2 hr. LPS of one hundred ng/mL was added for the final 20 min of culture at 37 °C under 5% CO_2-humidified atmosphere. The supernatants were collected by centrifugation at 14,000 × g for 10 min and stored at -70 °C until use for ELISA. The amount of TNF-α was determined by a direct sandwich ELISA using the Quantikine® canine TNF-α immunoassay kit (R&D Systems Inc, Minneapolis, MN, USA) according to the manufacturer's protocol. All samples, standards and controls were assayed in triplicate. The optical density was determined using an automated microplate reader (ELx808, Bio-Tek Instruments Inc, Winooski, Vermont, USA) at 450 nm with correction wavelength set at 540 nm. TNF-α was quantified from eight titration points using standard curves generated with purified canine TNF-α, and the concentrations were expressed as pg/mL. Lower and upper detection limits were 7.8 and 500 pg/mL, respectively.

Statistical analyses

All statistical analyses were carried out using SigmaStat version 2.0 Software (SPSS Inc, Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to determine differences between the control and each treatment group, followed by Dunnett's *post hoc* test. Comparisons of two groups were using the *t*-test. $P < 0.05$ was considered statistically significant. Data represents the mean ± standard deviation (SD).

Results

Effect of MPSS on the circulating leukocyte numbers

To examine the change of circulating leukocyte numbers in response to MPSS injection, complete blood counts were performed using an automated hematology analyzer. All of the total leukocyte and neutrophil numbers were significantly increased 2 hr ($P = < 0.001$), 12 hr ($P < 0.001$), and 24 hr ($P = 0.007$) after the completion of dosing, compared with the control group that received normal saline or the MPSS group before dosing (0 hr) ($P < 0.001$), and recovered 48 hr within reference range after the completion of dosing (Fig 1A and 1B), respectively. The monocyte numbers were also significantly increased 2 hr ($P = 0.002$), 12 hr ($P < 0.001$), and 24 hr ($P = 0.001$) after the completion of dosing, compared with the control group or the MPSS group before dosing (0 hr) ($P < 0.001$), and recovered within reference range 48 hr after the completion of dosing (Fig 1C). However, there were no significant differences in the numbers of lymphocyte, eosinophil, and basophil (Fig 1D, 1E, and 1F).

Effect of MPSS on the phagocytic capacity and OBA of canine PMNs and PBMCs

PMNs and PBMCs were collected before (0 hr) and 2, 12, and 24 hr after intravenous injection of MPSS and the phagocytic capacity and OBA were measured simultaneously. The phagocytic capacity of PMNs in dogs that received MPSS was significantly decreased 2 hr after the completion of dosing, compared with the MPSS group before dosing (0 hr) ($P < 0.001$) or the control group that received normal saline ($P < 0.001$), and recovered 12 hr after the completion of dosing (Fig 2A), to a level similar to the pre-dosing level (0 hr). The OBA of PMNs was significantly reduced 2 hr after the completion of dosing, compared with the MPSS group before dosing (0 hr) ($P < 0.001$) or the control group ($P = 0.003$), and recovered 12 hr after dosing (Fig 2B). The OBA of PBMCs in dogs that received MPSS was also decreased 12 hr, compared with the MPSS group before dosing (0 hr) ($P = 0.036$) or the control group ($P = 0.031$), and recovered 24 hr (Fig 2D). However, there was no statistically significant difference in the PBMCs phagocytic capacity of dogs that received MPSS (Fig 2C).

Effect of MPSS on LPS-induced TNF-α release from PMNs and PBMCs

Measurable TNF-α was not released in the absence of LPS. There was no significant change in the TNF-α release from canine PMNs treated with LPS (100 ng/mL) for 20 min *in vitro* after the completion of MPSS dosing (Fig 3A). However, LPS-induced TNF-α release from PBMCs was significantly decreased 12 hr after the completion of dosing, compared with the MPSS group before dosing (0 hr) ($P < 0.001$), and recovered 48 hr after the completion of dosing, to a level similar to the pre-dosing level (0 hr) (Fig 3B).

Discussion

In the present study, the MPSS dosing triggered an increase of total circulating leukocyte numbers including neutrophilia and monocytosis. The increase of total circulating leukocyte numbers was most likely correlated with neutrophila because the majority of leukocytes consisted of neutrophils. The neutrophilia causing by GC treatment is mainly results from increased bone marrow release of neutrophils, but it also reflects decreased migration of neutrophils from circulation into tissues as well as decreased adherence of neutrophils with a consequent shift of cells from the marginal neutrophil pool to the circulating neutrophil pool (22). The neutrophilia is typically without a left shift and accompanied by lymphopenia, eosinipenia, and monocytosis (35). The treatment with GCs also results in neutrophilia through stimulation of granulocyte colony stimulating factor and through delaying apoptosis (24). Therefore, we assumed that

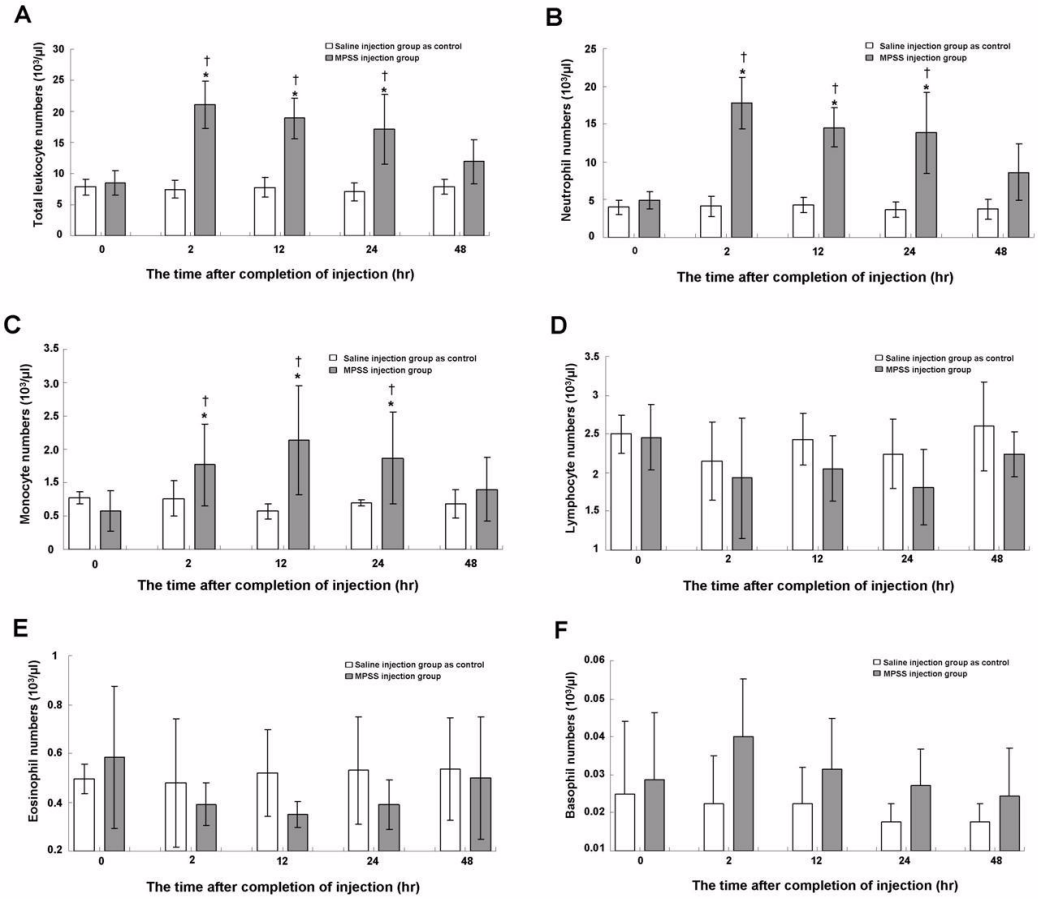

Fig 1. Effect of intravenous MPSS on the circulating numbers of canine peripheral blood leukocyte including total leukocytes (A), neutrophils (B), monocytes (C), lymphocytes (D), eosinophils (E), and basophils (F). At the indicated times after the conclusion of MPSS dosing, peripheral blood was collected, and peripheral blood leukocyte numbers were counted by an automated counter, as described in Materials and Methods. One-way ANOVA was used to investigate differences between control (0 hr) and each treatment group at the indicated times after dosing (2, 12, 24, and 48 hr), followed by post-hoc Dunnett's test. Differences between saline injection group and MPSS injection group at each of the indicated time were analyzed by *t*-test. Data represents the means ± SD of 6 different dogs. *$P < 0.05$ *vs* MPSS injection group at 0 hr (one-way ANOVA); †$P < 0.05$ vs. saline injection group at 2, 12, 24, and 48 hr, respectively (*t*-test).

application of MPSS protocol may induce not only the release of neutrophil from bone marrow and marginated pool but also delaying apoptosis, although there was no morphologic evaluation to determine the mature condition of leukocytes in this study. Otherwise, the changes of leukocyte numbers disappeared within 24 hr after all MPSS dosing. The rapid normalization on leukocyte numbers changed by MPSS may be due to the short half-life of MPSS, because MPSS is a synthetic glucocorticoid with a half-life of approximately 3 hr and a median duration of 1.25-1.5 days (27).

Traditionally, the properties of GCs as an immunosuppressive or anti-inflammatory agent are dependent on genomic mechanisms which are known as a course of the modulation of transcription, translation, and finally the synthesis of specific regulator proteins through the binding of GCs to its cytosolic receptor (40). It has been also well known that the the major effect of GCs in the phagocytic response is in blocking the interaction of phagocytes with foreign particles (3). In the present study, the application of high-dose MPSS protocol decreased both the phagocytic capacity and OBA of canine PMNs 2 hr after the completion of dosing, as shown in a previous report (20). These decreased phagocytic capacity and OBA by MPSS were rapidly recovered within 24 hr after the completion of dosing, indicating that the inhibitory effect of high-dose MPSS protocol is only presented within a short period relatively. However, it has been also reported that GCs can rapidly inhibit functions of mouse peritoneal macrophage *in vitro* through nongenomic mechanisms (25). The nongenomic GC effects have been known to compose of both specific nongenomic effect, which is mediated by membrane receptor (8) and nonspecific nongenomic effect, which

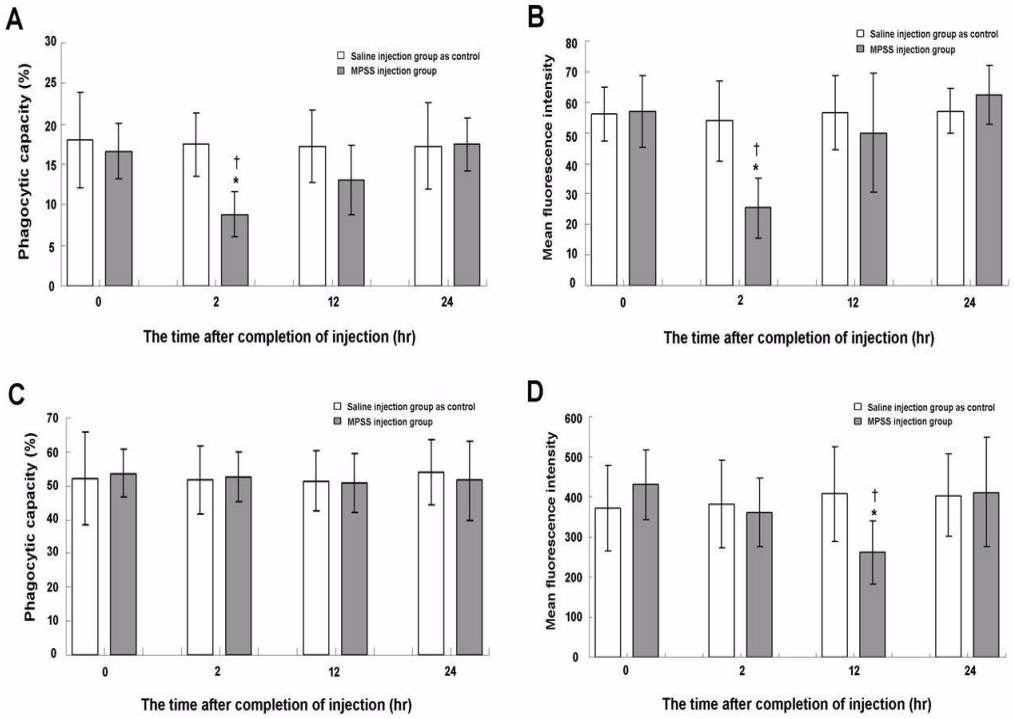

Fig 2. Effect of intravenous MPSS on the phagocytic capacity (A and C) and OBA (B and D) of canine PMNs (A and B) and PBMCs (C and D). At the indicated times after the conclusion of MPSS dosing, peripheral blood was collected, and PMNs and PBMCs were isolated and then cultured for 2 hr. Cultures (1×10^6 cells/mL per well) were supplemented with fluorescent microspheres (2×10^7 beads/mL per well) for the final 1 hr of culture, and with DHR-123 for the final 15 min, and the phagocytic capacity and OBA were simultaneously measured by flow cytometry, as described in Materials and Methods. One-way ANOVA was used to investigate differences between control (0 hr) and each treatment group at the indicated times after dosing (2, 12, and 24 hr), followed by *post hoc* Dunnett's test. Differences between saline injection group and MPSS injection group at each of the indicated time were analyzed by t-test. Data represents the means ± SD of 6 different dogs. *$P < 0.05$ *vs* MPSS injection group at 0 hr (one-way ANOVA); †$P < 0.05$ *vs* saline injection group at 2 hr (*t*-test).

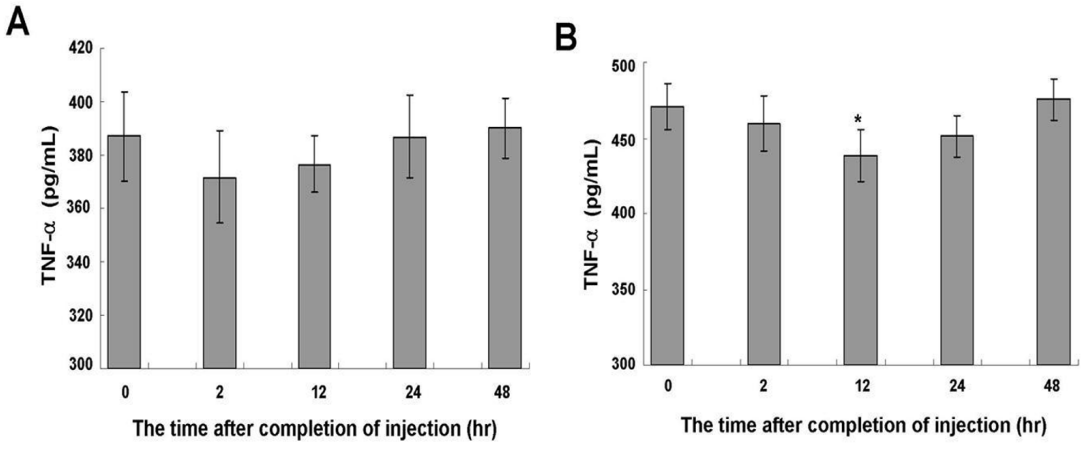

Fig 3. Effect of intravenous MPSS on TNF-α release from canine PMNs (A) and PBMCs (B). Freshly isolated PMNs and PBMCs (2×10^6 cells/mL per well) were incubated for 2 hr, respectively. LPS of one hundred ng/mL was added for the final 20 min of culture. The concentrations of TNF-α in the cultured supernatants were measured by a direct sandwich ELISA. One-way ANOVA was used to investigate differences between control (0 hr) and each treatment group at the indicated times after dosing (2, 12, 24, and 48 hr), followed by *post hoc* Dunnett's test. Data represents the means ± SD of 6 different dogs. *$P < 0.05$ *vs* MPSS injection group at 0 hr.

relates to a change in the membrane fluidity by physicochemical interactions with cellular membranes (10). The modulation of membrane fluidity may be an essential course for phagocytic procedure and important to uptake of particles by phagocytes (29). Therefore, we presumed that the inhibitory effect of high-dose MPSS on the phagocytic capacity and OBA of canine PMNs may be correlated to the rapid nongenomic mechanism of GCs such as a decrease of the membrane fluidity.

The MPSS dosing also decreased the OBA of PBMCs. However, its application did not have an inhibitory effect on the phagocytic capacity of PBMCs. One report is in agreement with this finding. Corticosterone could inhibit superoxide anion production but not phagocytosis by mouse peritoneal macrophages (25). In the present study, one possibility for no decrease of phagocytic capacity in PBMCs may be related to the population of phagocytes in PBMCs since canine PBMCs are composed of both approximately 30 % monocytes and 70 % lymphocytes. During phagocytosis, peripheral blood phagocytes increase their oxygen consumption through the activity of an NADPH-oxidase that generates superoxide anion and hydrogen peroxide (9). It is generally assumed that the NADPH-oxidase is assembled and activated either in the plasma membrane or in the membrane of an internalized phagosome (16). However, it has been reported that an intracellular activation of the oxidase is also achieved in the absence of a phagocytosed prey (21). Phagosome formation is not a prerequisite for superoxide production, because the NADPH oxidase may also be activated by soluble stimuli including phorbol 12-myristate 13-acetate (PMA) and N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (34). Consequently, the inhibitory MPSS effect on OBA of PMNs might be a result of the decreased phagocytic capacity of PMNs, but the inhibitory effect of OBA of PBMCs might be also due to a direct effect of MPSS on some enzyme system responsible for the respiratory burst.

Next, we asked whether the application of high-dose MPSS protocol have an effect on LPS-induced TNF-α release from canine peripheral blood phagocytes. The TNF-α release by LPS stimulation on PBMCs exposed to MPSS was suppressed, but its release from PMNs was not affected. The suppressive effect of MPSS on TNF-α release from PBMCs was rapidly disappeared within 48 hr. These results suggested that the canine PBMCs on LPS-induced TNF-α may be sensitive more than PMNs when exposed to high-dose MPSS. The genomic effect of GCs on production of proinflammatory cytokines such as TNF-α has been extensively studied, but the effect of MPSS on canine peripheral blood phagocytes has not been yet reported. GCs have been shown to suppress LPS-induced TNF-α production on monocytes/macrophage through the decrease of TNF-α gene transcription by blocking of nuclear factor (NF)-κB transcriptional activity (17,37). It has been reported that glucocorticoid-mediated suppression of TNF-α on peripheral blood monocytes was presented by administered single dose of prednisolone of 1.5 mg/kg to healthy humans (38). Also, dexamethasone suppressed transcription of TNF-α in promonocytic THP-1 cells between 90 min and 3 hr after LPS exposure (36). These findings indicated that the MPSS dosing may have a genomic effect on TNF-α production from canine peripheral blood PBMCs.

In conclusion, the present results suggest that high-dose MPSS can inhibit the phagocytic capacity and OBA of canine peripheral blood phagocytes and induce leukocytosis, neutrophilia, and monocytosis for relatively short period. Furthermore, its application may modulate TNF-α release from canine PBMCs. However, to clarify the nongenomic and genomic effects induced by the application of high-dose MPSS protocol, further researches on canine phagocytes will be necessary. Also, the treatment with MPSS should be applied with caution for patients with SCI because of an inhibitory effect on innate immune functions of canine phagocytes.

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