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Effect of Ketamine on the Oxidative Burst Activity of Canine Peripheral Blood Leukocytes *In Vitro*

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Abstract : Ketamine, one of general anesthetics for human and veterinary use, is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist which interferes with the action of excitatory amino acids. It has been reported to impair various leukocyte functions. In this study, the effect of ketamine on the oxidative burst activity (OBA) of canine peripheral blood leukocytes was examined. The OBA of canine peripheral blood phagocytes was analyzed by flow cytometry system. Ketamine at higher concentration such as 1,000 μM exhibited a low viability of leukocytes. Thus, ketamine was used at concentration of 10 to 500 μM showing no cytotoxic effect and high cell viability. The OBA of leukocytes in the presence or absence of latex beads was analyzed by addition of dihydrorhodamine 123. The direct treatment of ketamine revealed the inhibitory effect on the OBA of peripheral blood polymorphonuclear cells (PMN) and monocyte-rich cells but not peripheral blood mononuclear cells (PBMC) in the presence of latex beads. However, when latex beads were not added to PMN, its OBA was not inhibited by ketamine. The OBA of PMN and monocyte-rich cells but not PBMC in the presence of latex beads was also inhibited by culture supernatant from ketamine-treated- PBMC but not -PMN. But the OBA of PMN in the absence of latex beads was not inhibited by culture supernatant from PBMC treated with ketamine. Therefore, these results suggested that ketamine has the inhibitory effect on the OBA of canine peripheral blood phagocytes such as neutrophils and monocytes during phagocytic response.

Key words **:** ketamine, dog, oxidative burst activity (OBA), peripheral blood leukocytes.

Introduction

When phagocytes are activated by variety of foreign bodies, they are highly effective at generating reactive oxygen species (ROS) by a process known as the oxidative burst activity (OBA). In stimulated phagocytes, superoxide anion (O_2^-) is produced by a nicotinamide adenine dinucleotide phosphate reduced (NADPH) dependent oxidase. The leukocyte specific NADPH oxidase is a multicomponent enzyme responsible for agonist-dependent generation of ROS by phagocytes (3,6,24). Several microcidal oxidants such as hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) are generated from O_2^- (19). These ROS can be major source of damage to inflamed tissues (14). Phagocytes also possess nonoxidative mechanisms of killing that include an array of antimicrobial proteins and enzymes contained within a variety of cytoplasmic granules that are released into the phagosome upon ingestion (10).

Ketamine is 2(2-chlorophenyl)-2-(methylamino)-cyclohexanone, an arylcycloalkylamine. It is structurally related to phencyclidine (PCP) and cyclohexamine. Ketamine is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist which interferes with the action of excitatory amino acids (1) and short acting general anesthetic agent for human and veterinary use.

General anesthesics including ketamine may influence the host defense system. The release of cytokines by cultured mononuclear cells has been inhibited after exposure to various anesthetics including volatile anesthetics and intravenous anesthetics, e.g., ketamine (23,25). Ketamine could suppress lipopolysaccharide (LPS)-induced TNF-α production in the serum and could inhibit endotoxin-induced nuclear factor kappa B (NF-κB) *in vitro* (28,32). Further, ketamine has been reported to impair various leukocyte functions. In LPS-stimulated leukocytes, ketamine decreased cell adherence and suppressed oxidant expression and chemotactic activity of neutrophils (16,31).

The aim of this study is to examine the effect of ketamine on the OBA of canine peripheral blood innate leukocytes.

Materials and Methods

Animals

Male, clinically healthy three Beagle dogs born from the same mother, ages of approximately 3 years, were used as

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blood donors. The dogs were housed in room temperature (22±2), maintaining day (12 h) and night (12 h) rhythm. The dogs individually managed in cages and fed on pellet diet (Merry dog, Purina Korea, Seoul, Korea).

Peripheral blood mononuclear cells (PBMC) and peripheral blood polymorphonuclear cells (PMN) isolation

Peripheral blood drawn in heparinized tube from jugular vein was layered on the equal volume of Histopaque solution (specific gravity, 1.077; Sigma-Aldrich Co., St. Louis, MO, USA.) and centrifuged at 400×g for 40 min at room temperature. The resulting PBMC in interface between plasma and Histopaque 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 PBMC was composed of both approximately 30% monocytes and 70% lymphocytes when determined in cell counting by Wright-Giemsa staining. The PMN was obtained from layer of erythrocyte sediment after PBMC 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 37°C and washed 3 times with PBS. The purity of neutrophils in final PMN suspension exceeded 96% when determined by cytospin smear and Diff-Quik stain. The viability of PBMC and PMN determined by a trypan blue dye exclusion methods was always more than 96%. All cells were resuspended in RPMI 1640 medium (Sigma-Aldrich Co.) supplemented with 2 mM L-glutamine, 0.02 mg/ml of genta-micin and 5% fetal bovine serum (Gibco Co., Grand Island, NY, USA) and finally adjusted to 2×10^6 cells/ml.

Culture supernatant

The isolated PMN and PBMC at density of 2×10^6 cells/ml in a well of a 24-multiwell plate (Nunc Co., Naperville, IL, USA.) were incubated with concentrations of 10, 100 and 500 μM of ketamine (Ketamine 50^R, Yuhan, Korea) for 24 h at 37°C under 5% CO_2-humidified atmosphere. The supernatant was collected by centrifugation at 5,000×g for 30 min, filtered with 0.45 μm-pore size membrane filter and stored at -70°C until use for assay.

Analysis of oxidative burst activity

One hundred microliter of the cells adjusted to 1×10^7 cells/ml were added into each well of a 24-well plate. The PMN and PBMC were incubated with ketamine (10, 100 and 500 μM) or culture supernatant from PMN and PBMC treated with ketamine (10, 100 and 500 μM) for 12 h at 37°C under a 5% CO_2-humidified atmosphere, respectively. Twenty microliter of 1×10^9 beads/ml of carboxylated-modified polystyrene latex beads were added into each well for the final 1 h. And 1 μM of dihydrorhodamine 123 (DHR) (Sigma-Aldrich Co.) was added into each well for the final 15 min. The PMN and PBMC incubated without fluorescein isothiocyanate (FITC)-latex beads were used 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). After the third washing, the supernatant was discarded and replaced with 1 ml PBS. All incubation steps were carried out in a dark environment. Oxidative burst activity was measured by a flow cytometry (BRYTE HS, Bio-Rad Micro-science Ltd., Hertfordshire, UK). Rhodamine (green) fluorescence was measured on 5,000 cells per sample. To use the monocyte-rich cells in flowcytometric cytography of PBMC, they were fractioned by cell size of PBMC from dot plot profile in flowcytometric cytography (18). Similarly, rhodamine fluorescencein from 5,000 cells in monocyte-rich fraction was measured.

Data analysis

The Student's *t*-test was used for statistical significance determinations. All data were expressed as mean±standard error of means (S.E.M.).

Results

Cell viability of PMN and PBMC

PMN and PBMC were treated with 10 to 1,000 μM ketamine for 24 h. Ketamine at higher concentration such as 1,000 μM exhibited a low viability ($P<0.05$) of leukocytes when compared with those of untreated controls. Thereafter, ketamine was used at concentration of below 500 μM (Table 1).

Direct effect of ketamine on OBA of leukocytes.

To examine the direct effect of ketamine on OBA of leukocytes, PMN and PBMC at density of 1×10^6 cells/ml, respectively, were incubated with ketamine (10, 100 and 500 μM) for 12 h. The OBA of leukocytes was not affected by 10 μM of ketamine regardless of presence of latex beads. When latex beads were not added to PMN, there was no change on OBA

Table 1. Cell viability (%) of PMN and PBMC treated with 10 to 1,000 μM ketamine for 24 h

Cell type	Ketamine concentration (μM)				
	0	10	100	500	1,000
PMN (%)	98.67±1.40	98.67±1.2	98.03±0.75	97.83±0.68	96.00±0.79*
PBMC (%)	96.60±1.04	96.70±0.45	95.33±1.00	95.03±0.73	93.87±0.40*

The values represent mean±SEM (n=3)
*$P<0.05$, compared to control (0 μM ketamine).

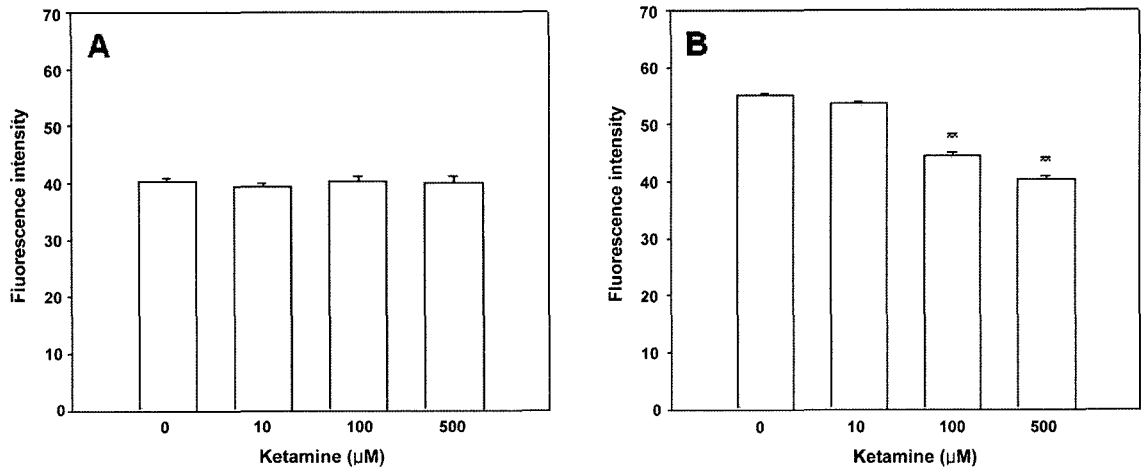

Fig 1. The direct effect of ketamine on OBA of PMN. PMN was treated with ketamine (10, 100 and 500 μM) for 12 h. **A**, PMN without latex beads. **B**, PMN phagocytized with latex beads. The values represent mean±SEM (n=3). $^{**}P<0.01$, compared to control (0 μM ketamine).

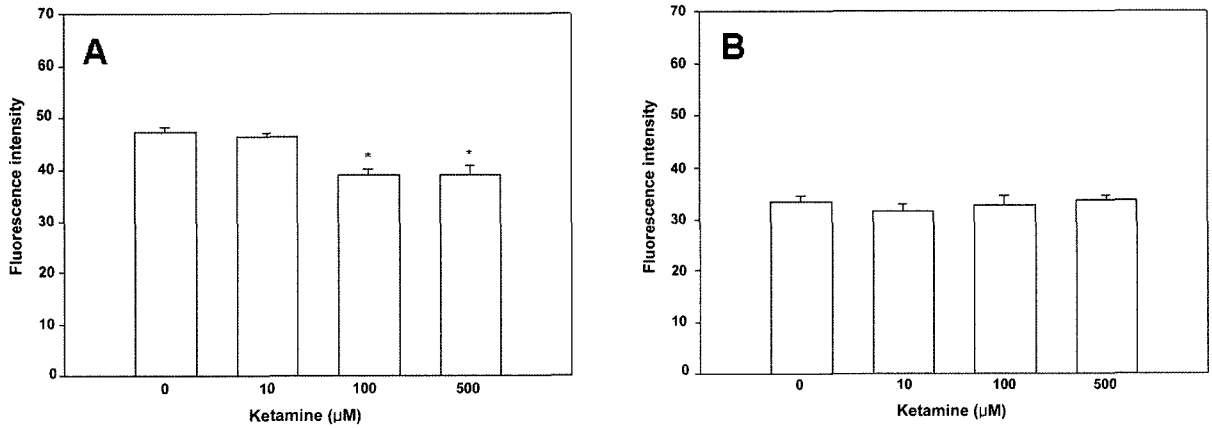

Fig 2. The direct effect of ketamine on OBA of monocyte-rich cells and PBMC in the presence of latex beads. Monocyte-rich cells and PBMC were treated with ketamine (10, 100 and 500 μM) for 12 h. **A**, monocyte-rich cells phagocytized with latex beads. **B**, PBMC phagocytized with latex beads. The values represent mean±SEM (n=3). $^{*}P<0.05$, compared to control (0 μM ketamine).

of PMN (Fig 1A). However, the direct addition of 100 and 500 μM of ketamine showed significant inhibitory effect ($P<0.01$) on the OBA of PMN (Fig 1B) in the presence of latex beads as compared with control without ketamine. The OBA of latex beads-phagocytized monocyte-rich cells (Fig 2A) but not -PBMC (Fig 2B) was also significantly inhibited ($P<0.05$) by direct treatment of ketamine (100 and 500 μM) as compared with control.

OBA of phagocytes by culture supernatant from leukocytes treated with ketamine

To examine the effect of culture supernatant from ketamine-treated PMN and ketamine-treated PBMC on OBA of phagocytes, PMN and PBMC at density of 1×10^6 cells/ml, respectively, were incubated with culture supernatant from PBMC and PMN treated with ketamine (10, 100 and 500 μM) for 12 h. Although the OBA of PMN in the absence of latex beads was not inhibited by culture supernatant from PBMC treated with 100 and 500 μM of ketamine (Fig 3A), its OBA in the presence of latex beads was significantly inhibited ($P<0.01$) by culture supernatant from PBMC treated with ketamine (Fig 3B). The OBA of monocyte-rich cells (Fig 4A) but not PBMC (Fig 4B) in the presence of latex beads was also significantly inhibited by culture supernatant from PBMC treated with 100 and 500 μM of ketamine when compared with control without ketamine. The OBA of PMN (Fig 5A), monocyte-rich cells (Fig 5B) and PBMC (Fig 5C) was not affected by culture supernatant from PMN treated with ketamine. In addition, the OBA of all leukocytes was not inhibited by culture supernatant from PMN or PBMC treated with a concentration of 10 μM ketamine (Fig 3-5).

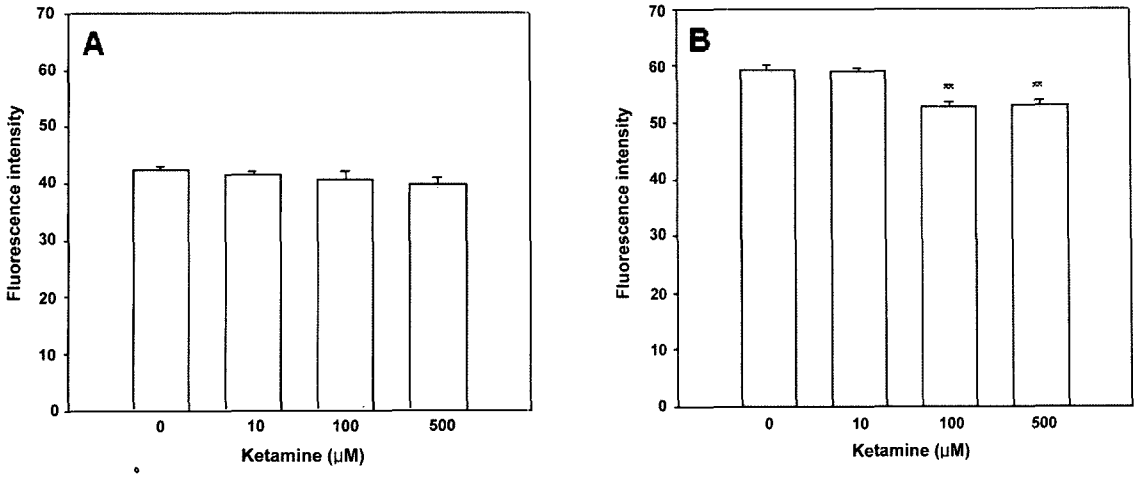

Fig 3. OBA of PMN by culture supernatant from PBMC treated with ketamine (10, 100 and 500 μM). **A**, PMN without latex beads. **B**, PMN phagocytized with latex beads. The values represent mean±SEM (n=3). **P<0.01, compared to control (0 μM ketamine).

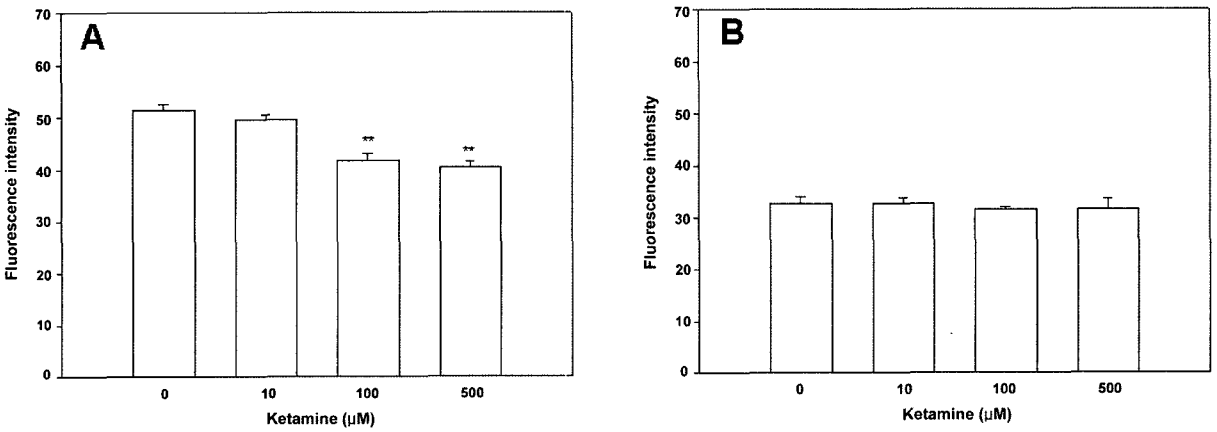

Fig 4. OBA of monocyte-rich cells and PBMC by culture supernatant from PBMC treated with ketamine (10, 100 and 500 μM). **A**, monocyte-rich cells phagocytized with latex beads. **B**, PBMC phagocytized with latex beads. The values represent mean±SEM (n=3). **P<0.01, compared to control (0 μM ketamine).

Discussion

In this study, ketamine at higher concentration, such as 1,000 μM, exhibited a low viability of leukocytess. However, ketamine at concentration of below 500 μM showed no cytotoxic effect and high cell viability. The 100 μM, a concentration of ketamine used in this study, is within the range of clinically relevance (8,13).

Treatment with at lower concentration of ketamine including culture supernatant from PBMC treated with 10 μM of ketamine did not influence the OBA of leukocytes. Further, the OBA of PMN and monocytes was inhibited by therapeutic concentration, 100 μM, of ketamine. PBMC might make no response on OBA due to a low ratio of monocytes because PBMC consists of approximately 70% lymphocytes and less than 30% monocytes in the dog. It is assumed that ketamine at lower concentration, such as 10 μM, may not have the important biological activity of peripheral blood leukocytes, e.g., cell adherence, migration, phagocytosis, and OBA.

Dihydrorhodamine 123 (DHR) has been shown to be the most consistent fluorescent probe for measuring OBA *in vivo* (9,26). The OBA is measured by the conversion of nonfluorescent compounds (dihydrorhodamine 123, DHR) to an oxidized green fluorescent product (rhodamine 123, RHO) by hydrogen peroxide via the stimulation of NADPH-oxidase (11). Stimulation of NADPH-oxidase is induced by latex beads. DHR freely permeates through cell membranes, whereas RHO tends to accumulate in mitochondria (27). In the present study, the OBA of phagocytes in the presence or absence of latex beads was analyzed by addition of DHR. There was no change on OBA of phagocytes in the absence of latex beads. But the OBA of phagocytes in the presence of latex beads was inhibited by

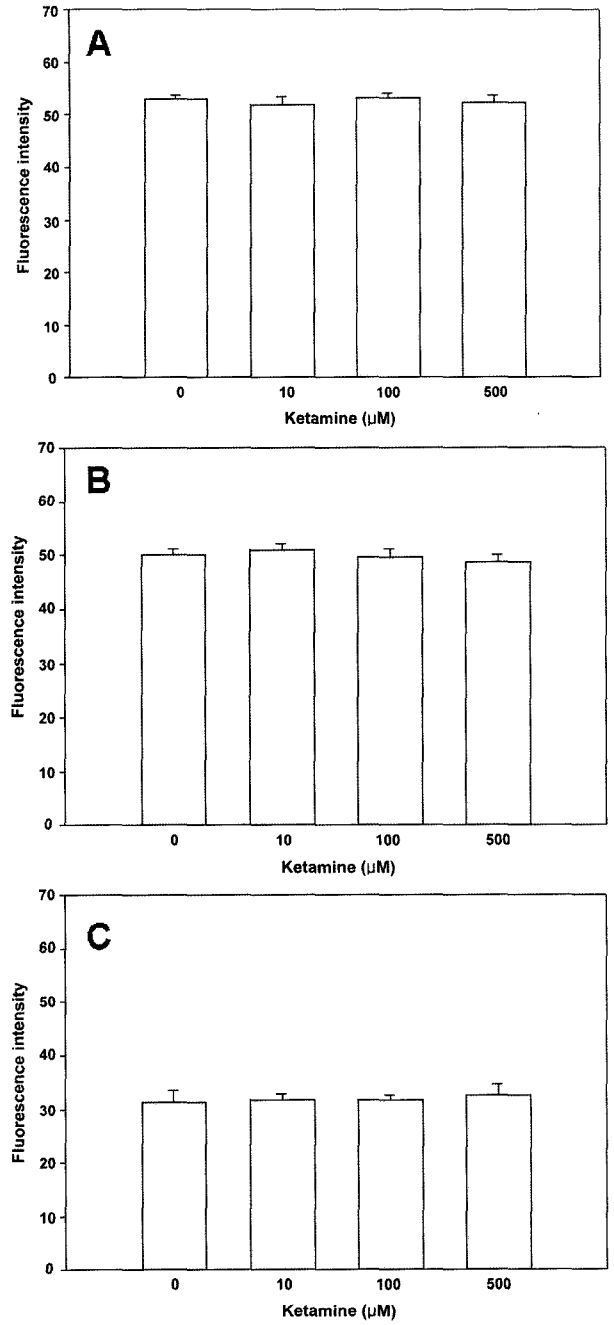

Fig 5. OBA of PMN, monocyte-rich cells and PBMC by culture supernatant from PMN treated with ketamine (10, 100 and 500 μM). **A**, PMN phagocytized with latex beads. **B**, monocyte-rich cells phagocytized with latex beads. **C**, PBMC phagocytized with latex beads. The values represent mean±SEM (n=3).

ketamine. These results indicated that the OBA of canine peripheral blood phagocytes can be measured only during phagocytic response. Activated phagocytes can produce and secrete large amounts of hydrogen peroxide, superoxide and nitric oxide for killing infective pathogens. Inhibition of OBA corresponds to a reduction in hydrogen peroxide production (30). This fact is suggested that a therapeutic concentration of ketamine is able to decrease oxidant production in phagocytes.

The direct addition of 100 and 500 μM of ketamine showed the inhibitory effect on OBA of PMN and monocyte-rich cells. Mitochondria are important energy-producing organelles and they convert energy into forms that are usable by the cell. Mitochondria are bounded by a double membrane and each of these membranes is a phospholipid bilayer with embedded proteins. Adenosine triphosphate (ATP), synthesized from the mitochondrial respiratory chain reaction, can enhance chemotactic migration and phagocytic ingestion of macrophages and neutrophils through the purinergic P2 receptor pathway or by elevation of intracellular Ca^{2+} (7,12,22). In murine polymicrobial sepsis, a decrease in cellular ATP levels was reported to be associated with marked suppression of the functions of lymphocytes and macrophages (2). A therapeutic concentration of ketamine can reduce the mitochondrial membrane potential (5). Depolarization of the mitochondrial membrane can lead to mitochondrial dysfunction that can induce inhibition of cellualr ATP synthesis, which contributes to phagocytic activity and OBA of phagocytes (17,20). Thus, the suppressive effect of ketamine on the OBA of phagocytes may be caused by the inhibition of ATP synthesis via the reduction of the mitochondrial membrane potential.

The OBA of PMN and monocyte-rich cells was inhibited by culture supernatant from PBMC treated with 100 and 500 μM of ketamine. The phagocytic activity and OBA of phagocytes are modulated by inflammatory cytokines including interlukin (IL)-1, IL-2, IL-8, interferons and tumor necrosis factors (TNFs) released from PBMC (4,29). Especially, TNF-α is proinflammatory and thus play a central role as mediators of the host response to infection and injury. It could increase neutrophil phagocytosis and superoxide generation (21). Inhibition of inflammatory cytokines production can also decrease the immune response. Several previous studies were reported that ketamine could suppress the expression of the inflammatory cytokines after endotoxin challenge (15,25). The suppressive effect of ketamine on the OBA of peripheral blood phagocytes by culture supernatant from PBMC treated with ketamine may be also associated with the decreased spontaneous expression of inflammatory cytokines such as TNF-α.

The overall results of this study suggested that ketamine has the inhibitory effect on the OBA of canine peripheral blood phagocytes such as neutrophils and monocytes during phagocytic response.

Acknowledgements

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In Vitro에서 개 말초혈액 백혈구의 순간산소과소비현상에 대한 케타민의 효과

김민준 · 강지훈 · 양만표[1]

요 약 : 전신마취제인 케타민은 흥분성 아미노산의 활성을 방해하는 N-methyl-D-aspartate (NMDA) 수용체의 비경쟁적인 길항제이다. 본 연구는 개 말초혈액 백혈구의 순간산소과소비현상(Oxidative burst activity; OBA)에 있어서 케타민의 효과를 검토하였다. 탐식세포의 OBA는 유세포 분석기로 분석하였다. 케타민을 말초혈액 다형핵백혈구(peripheral blood polymorphonuclear cells; PMN)와 monocyte-rich cells에 직접처리 하였을 때는 OBA가 감소하였으며, 또한 케타민을 처리한 말초혈액 단핵구세포(peripheral blood mononuclear cells; PBMC) 배양상층액에 의해서도 PMN과 monocyte-rich cells의 OBA가 감소하였다. 그러나 케타민을 처리한 PMN 배양상층액에 의해서는 탐식세포의 OBA에 있어서 아무런 변화가 없었다. 하지만 이러한 OBA의 감소는 latex beads를 넣어 탐식반응이 일어날 때만 측정되었다. 이상의 결과로부터 탐식반응이 일어나는 동안 케타민은 호중구와 단핵구와 같은 개 말초혈액 탐식구의 OBA에 있어 억제효과를 나타내었다.

주요어 : 케타민, 개, 순간산소과소비현상, 말초혈액백혈구