NOTE Internal Medicine

Changes in Leukocyte Population after Ozonated Autohemoadministration in Cows with Inflammatory Diseases

Hiromichi OHTSUKA1), Atsuya OGATA2), Nobuhiro TERASAKI3), Masateru KOIWA4) and Seiichi KAWAMURA1)

1)Department of Large Animal Internal Medicine, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034–8628, 2)Sohya Agricultural Mutual Relief Association, Esashi, Hokkaido 098–5205, 3)Kushiro Agricultural Mutual Relief Association, Tsurui, Hokkaido 085–1204 and 4)Department of Large Animal Clinical Sciences, Graduate School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069–8501, Japan

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ABSTRACT. In this study, we investigated whether ozonated autohemoadministration (OAHA) influences leukocyte populations in cows with clinical inflammatory disease. Eleven cows with inflammatory disease (Inflammatory Group) and three healthy cows (Control Group) were used for this study. The CD4+/CD8+ ratio in the Inflammatory Group increased significantly compared to that in the Control Group 3 to 4 days after OAHA treatment. In the Inflammatory Group, the number of CD14+ cells decreased gradually after OAHA, but CD14+ levels remained stable in the Control Group. The number of MHC class-II+ cells decreased gradually in the Inflammatory Group, but increased gradually in the Control Group, and the difference between the groups was significant on day 14 after OAHA. These findings suggest a possible difference in the activation of immune response after OAHA in infected cows compared to healthy cows.

KEY WORDS: dairy cow, leukocyte population, ozonated autohemoadministration.


Clinical cases of inflammatory disease are found frequently in lactating cows, and these cases are generally treated by administration of antibiotic drugs. However, social awareness of food safety issues involving livestock treated with antibiotics has grown in recent years [5], and as a result, veterinarians need to investigate alternative treatments for inflammatory diseases that do not rely on antibiotics. Depression of immune function has been observed in cows with inflammatory disease [15, 16]. Therefore activation of the immune response is one possible goal for alternative therapies.

Ozone is known as a toxic gas to humans and animals [10, 11]. Recently, however, the use of ozone therapy for humans has been reported to be effective for a variety of viral infectious diseases [6, 7]. Medical ozone exposure therapy is effective for patients with sluggish purulent inflammations of maxillofacial soft tissues due to the induced activation of leukocyte phagocytic activity [20]. In bovine veterinary medicine, ozone therapy has also been useful in the treatment cows with acute clinical mastitis [13].

The biological effects of ozone therapy are the result of immunological activation, including the production of cytokines by leukocytes [2, 3, 9]. Although ozone therapy has been reported to be effective in humans, the immune response to ozonated autohemotherapy has not been clarified in vivo, and the effect of ozonated autohemotherapy on peripheral leukocyte populations remains largely unknown in humans. Previously, we reported that ozonated autohemoadministration (OAHA) induced an increase of peripheral T cell and serum IL-6 activity in healthy calves [19]. But there have been no studies of peripheral lymphocyte populations after OAHA in cows with inflammatory diseases such as mastitis or arthritis. The aim of the present study was to investigate the influence of ozonated autohemotherapy on the immune response in cows with inflammatory disease by analyzing peripheral leukocyte populations after experimental OAHA administration.

Eleven cows with inflammatory disease, mastitis (N=5), arthritis (N=4), sole ulcer (N=2), and clinically healthy cows (N=3), weighing 470 to 630 kg, aged 3 to 6 years, were used in this study. Clinical signs for mastitic cows were induration of the mammary gland and mammary gland secretions that contain flakes and clots. All arthritis was caused by infected bacteria, and claudication and tumefaction of the diseased areas were observed. In the cows with a sole ulcer, serious claudication and ache sign were observed. All cows were treated with OAHA, and the experimental animals were divided into two groups, the Inflammatory Group (N=11) and the Control group (N=3). The experimental cows were given full measures of hay and water during the study period. They were all treated according to the Laboratory Animal Control Guidelines of Rakuno Gakuen University.

Blood samples were collected from the jugular vein of cows before OAHA and at 1, 2, 3, 4, 5, 7, and 14 days after OAHA treatment. Temperature, pulse, respiration, hematocrit value, and concentrations of total protein, albumin, and globulin were observed before OAHA. Whole blood samples were collected in tubes containing EDTA-2K. Total white blood cell counts (WBC) were measured by a blood cell counter. The absolute leukocyte count was calculated by multiplying the WBC by the observed percentage of lymphocytes or monocytes from the different count. Concentrations of albumin and globulin were measured by electrophoresis.

Ozone was generated from medical grade oxygen filtered using an OT-31ST-M1 ozone generator (Nippon Ozone,
Co., Ltd., Tokyo, Japan), which allowed the gas flow rate and ozone concentration to be controlled. Tygon polymer tubing and polypropylene disposable syringes were used throughout the reaction procedure to insure containment of the ozone and consistency in concentration. A blood volume of 25 ml to 100 ml was insufflated with ozone (10 µg/g) in an infusion bag to control the overall ozone concentration (20 µg/kg body weight).

Leukocyte populations were determined using a single fluorescein. Two ml of blood were mixed with 4 ml of 0.83% ammonium chloride solution, and the leukocytes were separated. Leukocyte samples were washed twice in phosphate-buffered saline (PBS). About 2 were separated. Leukocyte samples were washed twice in 0.83% ammonium chloride solution, and the leukocytes were suspended in PBS, and the samples were incubated at 4°C for 60 min in PBS containing monoclonal antibodies of bovine cell surface marker. The primary antibodies were lineage-specific monoclonal antibodies, including CACT83B (T-helper lymphocyte, CD4 antigen), 9ACT80C (T-cytotoxic/suppressing lymphocyte, CD8 antigen), MY4 (monocyte, CD14 antigen, Coulter Immunology, Hialeh, Florida, U.S.A.), and TH14B (monocytes or B cells, MHC class-II antigen, VMRD, Pullman, WA, U.S.A.). After 60 min incubation, the cells were washed twice in PBS, and incubated in PBS with the secondary antibody, which was FITC-conjugated goat anti-mouse IgG-fluorescein isothiocyanate (Cappel, Durham, NC, U.S.A.) for 30 min. After incubation, the samples were washed twice with PBS, and then cell surface markers were visualized with goat anti-mouse IgG. Cells were analyzed with a flow cytometer (EPICS ELITE ESP, Becton Coulter Inc., Florida, U.S.A.). Data was acquired from 5000 events per sample. In all analyses, mononuclear cells were gated out from other leukocyte population based on the dotplots of their forward scatter (FSC) and side scatter (SSC).

Data were assessed by Student’s t-test or Friedman test, and differences were judged to be significant at p<0.05.

In the blood analysis before OAHA, there were significant differences in some parameters between the two groups. Hematocrit value, albumin level, and A/G rate were significantly lower in the Inflammatory Group than in the Control Group. Significantly higher values of plasma protein, serum protein, alpha-globulin, and gamma-globulin were seen in the Inflammatory Group compared to the Control Group (Table 1).

WBC was higher in the Inflammatory Group than in the Control Group during the experimental period. There were no marked changes of WBC after OAHA in either of the two groups (Fig. 1). In the Inflammatory Group, the number of CD4+ T cells increased gradually after OAHA, and this difference became significant on day 3 compared to the pre-administration level. The number of CD4+ T cells in the Inflammatory Group remained low compared to the Control Group after OAHA and was significantly higher on days 3 and 4 compared to the pre-administration level. The CD4+/CD8+ ratio in the Inflammatory Group peaked on day 4 and was significantly higher than that in the Control Group. The CD4+/CD8+ ratio increased gradually in the Inflammatory Group after OAHA and was significantly higher on days 3 and 4 compared to the pre-administration level. The CD4+/CD8+ ratio in the Inflammatory Group peaked on day 4 and was higher than that in the Control Group from days 2 to 14 (Fig. 2). In the Inflammatory Group, the number of CD14+ cells was lower on days 4 to 14 after OAHA, and the difference was significant on day 4 compared with the level before OAHA treatment. The number of CD14+ cells was
LEUKOCYTE POPULATION AFTER OAHA IN INFLAMMATORY COWS

The number of MHC class-II + cells decreased in the Inflammatory Group, but increased in the control group after day 2. The number of MHC class-II + cells was lower in the Inflammatory Group than in the Control Group on days 3 to 14, and the difference between the two groups was significant at day 14 (Fig. 3). There was no marked alteration of clinical signs in any of the experimental cows after OAHA (data not shown).

With regard to peripheral leukocyte population, this study showed an increased CD4+/CD8+ ratio and a decreased CD14+ level after OAHA in the cows with inflammatory disease. Markable hypergammaglobulinemia and hyperleukocytosis were observed in the Inflammatory Group before OAHA, and this finding has also been reported in cows with chronic inflammatory disease. This immunity abnormality before OAHA administration in diseased cows may influence the changes in leukocyte population caused by ozonated blood stimulation.

The present study found a significant increase in the CD4+/CD8+ ratio due to increased CD4+ T cell numbers or decreased CD8+ T cell numbers in the Inflammatory Group after OAHA. In previous studies of cows with inflammatory disease, the CD4+/CD8+ cell ratio also increased [14], and increased numbers of CD4+ T cells or increased IL-2 and IL-4 production were observed in mastitic cows [12, 18]. The increased CD4+/CD8+ ratio might be a result of activated T cell function involving cytokines following OAHA stimulation. It appears reasonable to conclude that the response of T cells to OAHA in cows with inflammatory disease may differ from that in healthy cows.

The percentage of CD14+ monocytes reportedly decreases in patients with infectious disease [17]. One of the causes of decreased peripheral monocytes was migration of monocytes induced by cytokines or growth factors [4, 8]. Ozonation of peripheral blood mononuclear cells was performed to induce inflammatory responses, such as cytokine production [3]. On the other hand, cytokine induced downregulation of CD14+ expression on monocytes. These find-
ings suggested that the decrease in peripheral CD14+ cells in the Inflammatory Group might be caused by monocytes activated by OAH.

Previously, we reported increased B cells after OAH in healthy calves following increased serum IL-6 activity [19]. Larini and Bocci [9] reported that exposure to ozone induced marked IL-4 production in isolated peripheral blood mononuclear cells, and discussed a significant relationship between cytokine production and ozone concentrations. These findings suggested that there was an ozonated autohemo-activated humoral immunity response rather than a cellular immunity response in healthy animals. In this study, however, peripheral MHC class-II+ cell numbers decreased in the infected group after OAH, whereas these cells increased in the healthy controls. These changes in the Inflammatory Group after OAH may, in part, be due to an activated cellular immunity response.

In conclusion, the data obtained in the present study showed a significant difference in changes of lymphocyte population between the inflammatory and control groups after OAH, and this process appears to be related to inflammatory response. Bocci [1] reported that exposure of blood to appropriate ozone concentrations is effective in humans and that neither acute nor chronic side effects have ensued in millions of patients treated with ozonated autohemotherapy. It is possible that an unexpected immune response is induced by ozonated autohemotherapy in cows with inflammatory disease. Therefore, specific studies investigating the effect of OAH on immune response must be designed to determine whether ozonated autohemotherapy is effective in activating the immune system in cows with inflammatory diseases.

REFERENCES