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The Effects of Formulation on the Immunostimulatory Activity of Dihydroheptaprenol*

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ABSTRACT

Holstein steer calves received a single injection of Miglyol® (Sasol Chemical Industries, Ltd., Homburg, Germany) subcutaneously as a placebo, dihydroheptaprenol (DHP) (4 mg/kg) emulsified with lecithin subcutaneously, DHP in solution in Miglyol® (4 mg/kg) subcutaneously, or DHP in solution in Miglyol® (4 mg/kg) intranasally. The DHP emulsified in lecithin emulsion administered subcutaneously caused a substantial increase in body temperature, total leukocyte count, total neutrophil count, neutrophil cytochrome-*c* reduction, and neutrophil iodination 24 hours after administration and, for some of the parameters, at 48 hours. The DHP formulation in Miglyol® did not have any of these effects when administered subcutaneously or intranasally. The carrier and formulation of DHP apparently have major effects on the biologic activity of DHP.

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INTRODUCTION

Dihydroheptaprenol (DHP), a chemically synthesized polyprenol derivative, reportedly can enhance neutrophil function. Dihydroheptaprenol microemulsified with lecithin has been shown to increase the number and bactericidal activity of neutrophils when injected intramuscularly into cows and calves[1,2] and miniature pigs,[3] enhance alveolar macrophage-oxidative metabolism when injected intramuscularly into pigs,[4] and promote resistance to various bacterial infections in mice.[5] The DHP emulsion in lecithin can also enhance the

resistance of calves to intratracheal challenge with *Pasteurella haemolytica*.[6]

The objectives of this study were to determine the influence of two different formulations of DHP on neutrophil numbers and function in cattle when administered subcutaneously and to compare the subcutaneous and intranasal routes of administration of one of the formulations. The parameters included total and differential leukocyte counts, rectal temperature, and six neutrophil functions (i.e., random migration, chemotaxis, *Staphylococcus aureus* ingestion, cytochrome-*c* reduction, iodination, and antibody-dependent cell-mediated cytotoxicity [ADCC]).

MATERIALS AND METHODS

Experimental Design

Thirty-two Holstein steer calves weighing less than 160 kg each were the subjects of the study. Eight animals served as controls and received a placebo (Miglyol® 812 vehicle [Sasol Chemical Industries, Ltd., Homburg, Germany]) subcutaneously, eight animals received DHP prepared as a microemulsion with lecithin at 1% concentration (10 mg/ml) 4 mg/kg subcutaneously, eight animals received DHP in solution in Miglyol® 810 at 15% concentration 4 mg/kg subcutaneously, and eight animals received DHP in solution in Miglyol® 810 at 15% concentration 4 mg/kg intranasally. The Eisai Co., Ltd. (Tokyo, Japan) manufactured the DHP emulsion with lecithin. Pharmacia & Upjohn (Kalamazoo, MI) supplied the DHP and Miglyol® formulations. All animals received only one treatment dose and were treated on the same day. Immune function parameters were evaluated on two consecutive days before treatment and four consecutive days beginning one day after treatment. All treatments and sample collections began at approximately 8:00 AM. Rectal temperatures were recorded each day that blood samples were drawn and on the day of treatment.

The four groups of calves were housed together and fed free-choice alfalfa hay and an appropriate amount of grain mix each day, depending on body weight. Water was provided ad libitum from an automatic waterer in the pen. The calves were ear-tagged for individual identification.

Evaluation of Neutrophil Function

Blood (50 ml) was collected by jugular venipuncture into acid citrate dextrose anticoagulant. After centrifugation, plasma and buffy coat were discarded. Neutrophils were isolated from the packed red blood cells (RBCs) by hypotonic lysis of the RBCs, as previously described in the literature.[7] Isolated neutrophils were suspended in 0.015M phosphate-buffered saline solution at a concentration of 5.0×10^7 cells/ml for use in the function assays.

Neutrophil function assays were conducted as described in the literature.[7–9] Briefly, random migration under agarose was measured after an incubation period of 18 hours; the area of random migration was reported in square millimeters. Chemotaxis was measured by migration under agarose toward zymosan-activated serum; the chemotactic index was determined by dividing the distance of directed migration by the distance of random migration. Phagocytosis was measured using antibodycoatediododeoxyuridine(^{125}I)-labeled *S. aureus*. Neutrophils were incubated for 10 minutes with bacteria at a ratio of 60:1 (bacteria:neutrophils), then lysostaphin was added to remove the extracellular *S. aureus*; results were reported as percent of bacteria ingested. Reduction of cytochrome-*c*, a measure of superoxide anion production, was evaluated after a 5-minute incubation of neutrophils with cytochrome-*c* and opsonized zymosan. Results were reported as optical density/1.25×10^6 neutrophils/5 min. The

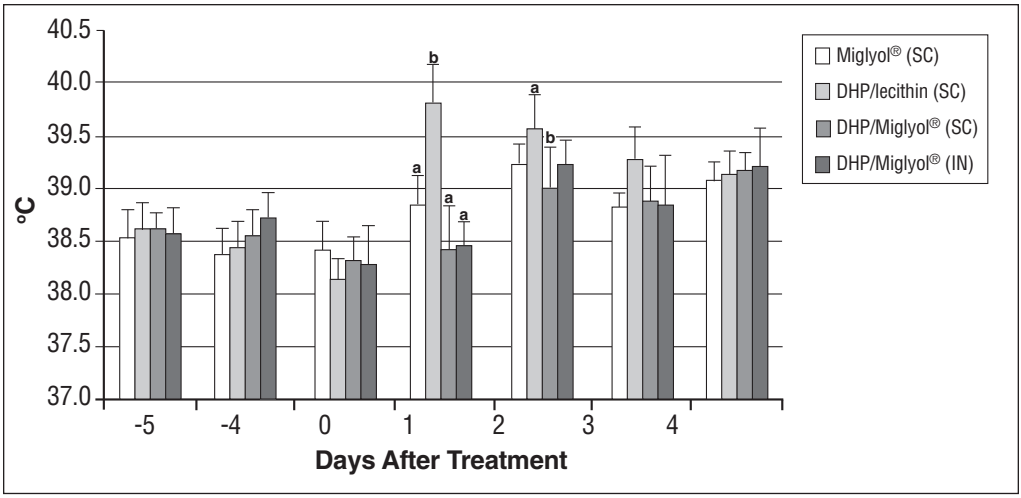

Figure 1. *Mean (± SEM) body temperature (°C) for steers treated with different formulations of DHP. Values with different letters* (a, b) *on the same day are significantly different (*P < *.05). DHP = dihydroheptaprenol; IN = intranasally; SC = subcutaneously; SEM = standard error of the mean.*

iodination reaction, a measure of the myeloperoxidase–hydrogen peroxide–halide system, was determined by incubating neutrophils with opsonized zymosan and sodium (Na) ^{125}I for 20 minutes; the reaction was terminated by the addition of trichloroacetic acid. Results were reported as nanomoles of NaI/10^7 neutrophils/hr. Antibody-dependent cell-mediated cytotoxicity was evaluated using antibody-coated ^{51}chromium-labeled chicken RBCs as the target cells. The effector:target-cell ratio was 10:1, and results were reported as percent of specific release during a 2-hour incubation.

Hematology

Blood samples for hematologic evaluation were collected in evacuated blood collection tubes containing sodium EDTA. Total and differential leukocyte counts were determined using an electronic particle counter and automated differential reader by the Clinical Pathology Laboratory at the Iowa State University College of Veterinary Medicine, Ames.

Statistics

Data were evaluated by analysis of variance using a least significant difference procedure, comparing individual groups each day to determine treatment effects. $P < .05$ was interpreted as being statistically significant.

RESULTS

Mean (± standard error of the mean) rectal temperatures are shown in Figure 1. The group receiving DHP emulsified in lecithin subcutaneously had significantly ($P < .05$) elevated rectal temperatures (39.7 ± 0.2°C) 24 hours after administration. By 48 hours after administration, the rectal temperatures in this group were no longer significantly elevated compared with the other groups. The groups receiving DHP in Miglyol® subcutaneously or intranasally did not have significantly elevated body temperatures compared with the group receiving Miglyol® only.

The group that received DHP emulsified in lecithin subcutaneously had significant ($P <$ .05) elevations in total leukocyte counts attrib-

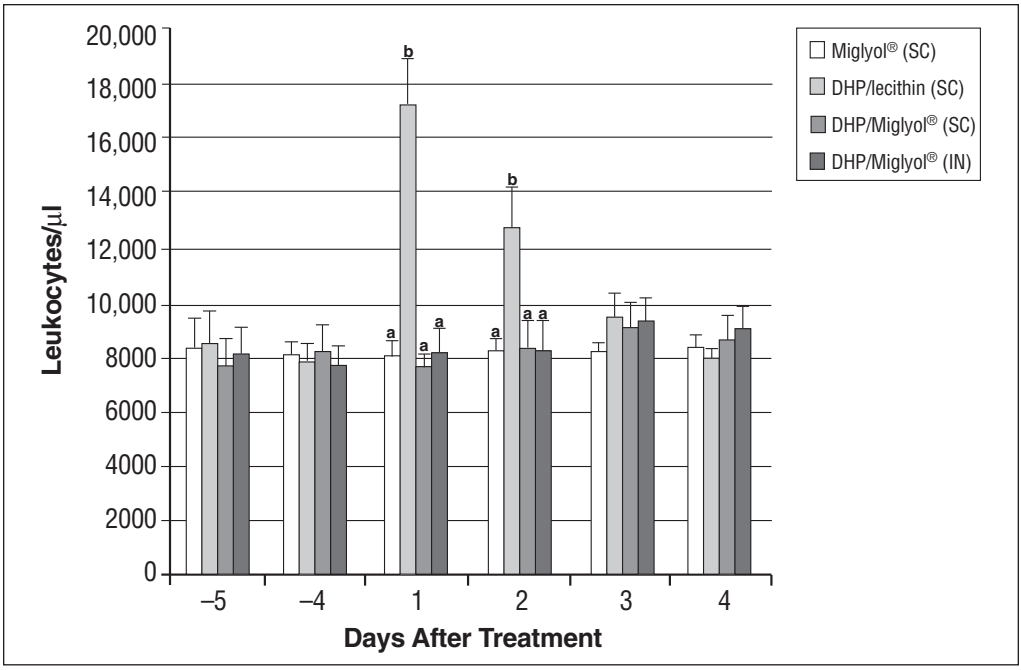

Figure 2. *Mean (± SEM) total white blood cell counts for steers treated with different formulations of DHP. Values with different letters* (a, b) *on the same day are significantly different (*P < *.05). DHP = dihydroheptaprenol; IN = intranasally; SC = subcutaneously; SEM = standard error of the mean.*

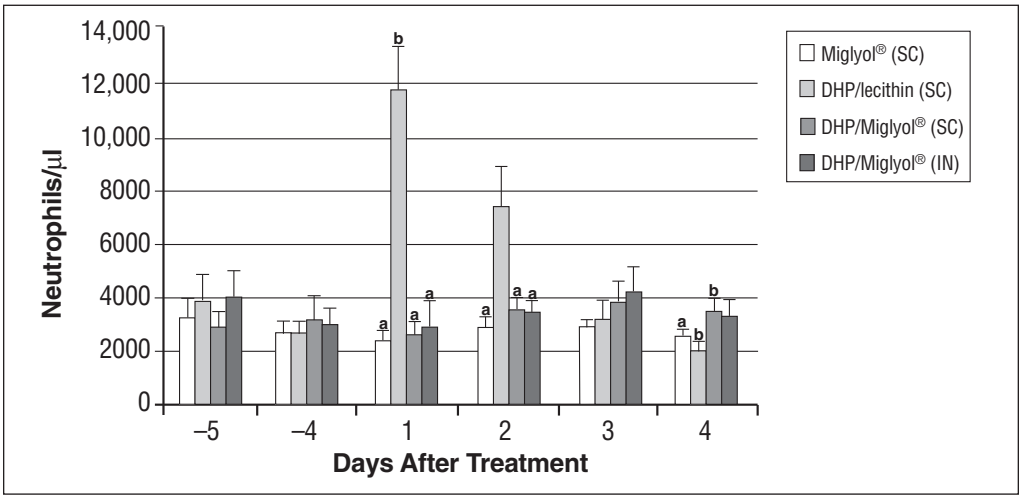

Figure 3. *Mean (± SEM) total neutrophil counts for steers treated with different formulations of DHP. Values with different letters* (a, b) *on the same day are significantly different (*P < *.05). DHP = dihydroheptaprenol; IN = intranasally; SC = subcutaneously; SEM = standard error of the mean.*

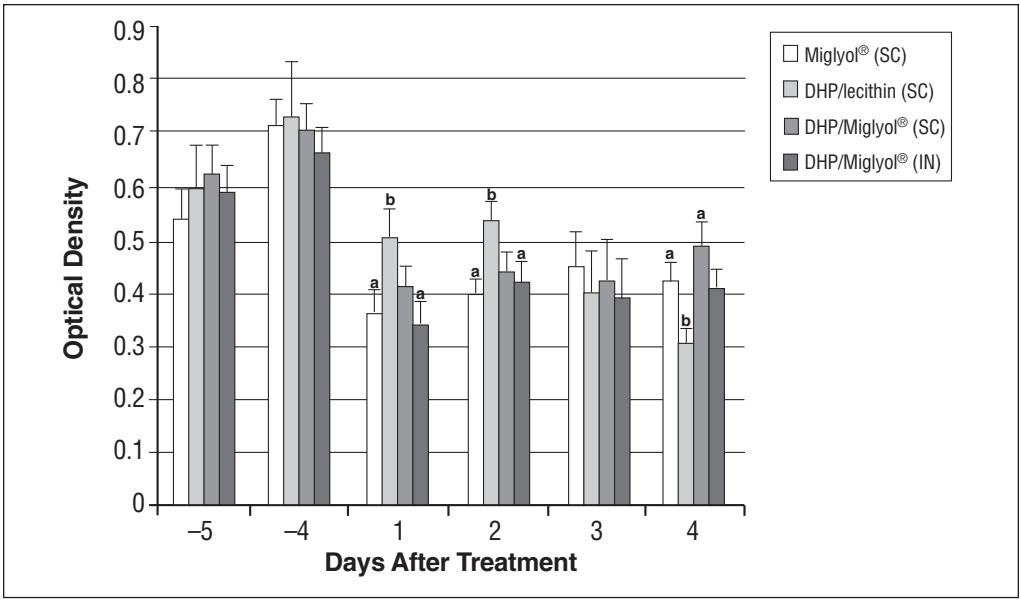

Figure 4. *Mean (± SEM) cytochrome-*c *reduction by neutrophils for steers treated with different formulations of DHP. Values with different letters* (a, b) *on the same day are significantly different (*P < *.05). DHP = dihydroheptaprenol; IN = intranasally; SC = subcutaneously; SEM = standard error of the mean.*

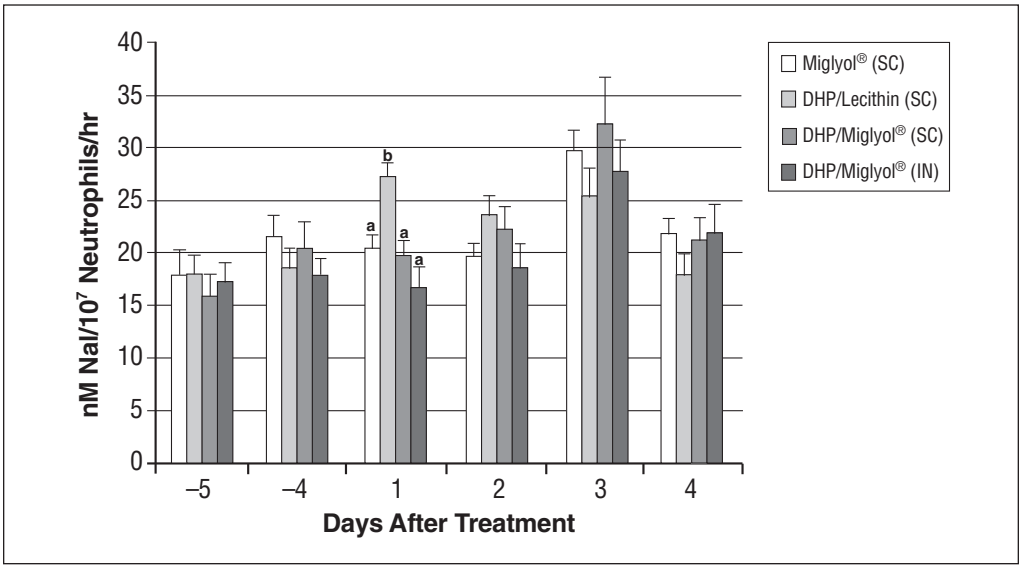

Figure 5. *Mean (± SEM) neutrophil iodination for steers treated with different formulations of DHP. Values with different letters* (a, b) *on the same day are significantly different (*P < *.05). DHP = dihydroheptaprenol; IN = intranasally; SC = subcutaneously; SEM = standard error of the mean.*

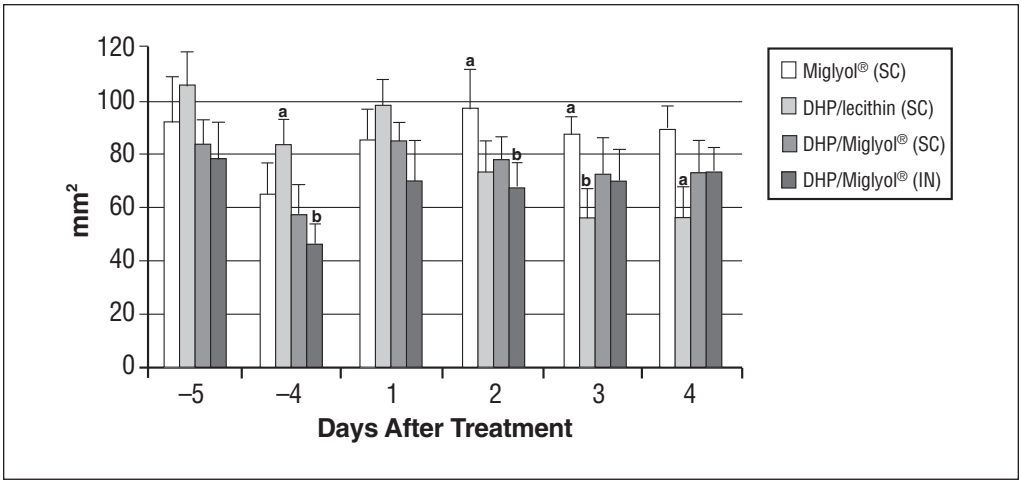

Figure 6. *Mean (± SEM) neutrophil random migration under agarose (mm²) for steers treated with different formulations of DHP. Values with different letters* (a, b) *on the same day are significantly different (*P < *.05). DHP = dihydroheptaprenol; IN = intranasally; SC = subcutaneously; SEM = standard error of the mean.*

utable to elevated neutrophil numbers 24 and 48 hours after treatment (Figures 2 and 3). None of the other groups had a tendency toward increased total leukocyte or total neutrophil counts. None of the treatments had significantly ($P < .05$) affected total lymphocyte, monocyte, eosinophil, or basophil counts during the first 48 hours after administration (data not shown).

The group that received DHP/lecithin emulsion subcutaneously had considerably enhanced neutrophil cytochrome-*c* reduction 24 and 48 hours after treatment but significantly suppressed cytochrome-*c* reduction 96 hours after treatment (Figure 4). The group receiving DHP emulsified in lecithin also had significantly ($P < .05$) enhanced neutrophil iodination 24 hours after treatment (Figure 5). The neutrophil iodination returned to normal limits by 48 hours after treatment. The DHP formulation in Miglyol® did not cause significant alteration in neutrophil iodination.

There was a tendency in all three groups receiving DHP to have reduced neutrophil random migration compared with the Miglyol® group at 48, 72, and 96 hours after treatment. This difference was statistically significant ($P < .05$) in the group receiving DHP in Miglyol® intranasally 48 hours after treatment and in the group receiving DHP emulsified in lecithin 72 and 96 hours after treatment (Figure 6). There was no significant influence of any of the three DHP formulations on neutrophil chemotactic index, the ability of neutrophils to ingest *S. aureus*, or ADCC by neutrophils (data not shown).

DISCUSSION

The DHP emulsified in lecithin caused significant ($P < .05$) increases in body temperature, total leukocyte count, total neutrophil count, neutrophil cytochrome-*c* reduction, and neutrophil iodination 24 hours after administration and, for some of these parameters, at 48 hours. Cytochrome-*c* reduction is a measure of the production of superoxide anion by neutrophils. The production of superoxide anion is an important first step in neutrophil oxidative-killing mechanisms. The iodination reaction is a measure of neutrophil myeloper-

oxidase–hydrogen peroxide–halide killing activity. Increases in neutrophil cytochrome-*c* reduction and iodination indicate enhanced neutrophil bactericidal capacity. When administered to pigs, DHP emulsified in lecithin has previously been shown to enhance alveolar macrophage-oxidative metabolism.[4] It may therefore be interesting to compare the influence of these DHP formulations on macrophage function. The DHP formulation in Miglyol® did not have the effects that DHP emulsified in lecithin had on neutrophil numbers or function, thus indicating that either Miglyol® blocks DHP activity or lecithin contributes to the activity observed directly or through a synergistic effect with DHP. It is also possible that the DHP emulsified in lecithin contained endotoxin or another contaminant that induced production of proinflammatory cytokines. However, the DHP/lecithin formulation was reportedly tested by the manufacturer and found to be negative for pyrogens.

The increased body temperature indicates that proinflammatory cytokines are probably induced by the DHP emulsified in lecithin. These cytokines may be directly responsible for alterations in neutrophil numbers and function. Various cytokines have been shown to influence bovine neutrophil function[10] and promote resistance to bacterial challenge.[11] Intramuscular administration of the DHP/lecithin formulation to calves (4, 6, or 8 mg/kg) and adult cows (1 or 2 mg/kg) had previously been shown to increase the number of neutrophils in blood, enhance their phagocytic killing activity toward *S. aureus,* and increase nitroblue tetrazolium reduction by neutrophils.[1,2] Nitroblue tetrazolium and cytochrome-*c* reduction measure production of superoxide anions by stimulated neutrophils. Therefore, the results reported here for the DHP/lecithin formulation are completely consistent with previously reported results. However, the biologic activity observed may not be attributable solely to the DHP component of the emulsion.

ACKNOWLEDGMENT

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