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THE EFFECT OF ASCORBIC ACID AND L-HISTIDINE THERAPY ON ACUTE MAMMARY INFLAMMATION IN DAIRY CATTLE

By

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ABSTRACT

THE EFFECT OF ASCORBIC ACID AND L-HISTIDINE THERAPY ON ACUTE MAMMARY INFLAMMATION IN DAIRY CATTLE

By

Anantachai Chaiyotwittayakun

Eight, non-pregnant Holstein cows with endotoxin-induced mastitis were selected to determine the effects of intravenous administration of L-histidine (L-His) and ascorbic acid (AA) by conducting in the Latin square crossover design. Repeated measurement analysis (SAS) was used to compare cows with an individual treatment groups; control, AA only, L-His only, and AA plus L-His by testing rectal temperature, milk production, somatic cell count, milk IgG_1, antioxidant activities, heart rate, respiratory rate, ruminal contraction rate and dry matter intake. AA treatments has a beneficial potential effect to increase recovery of milk production, and help to maintain DMI. However, both AA & L-His were not affected heart and respiratory rate.

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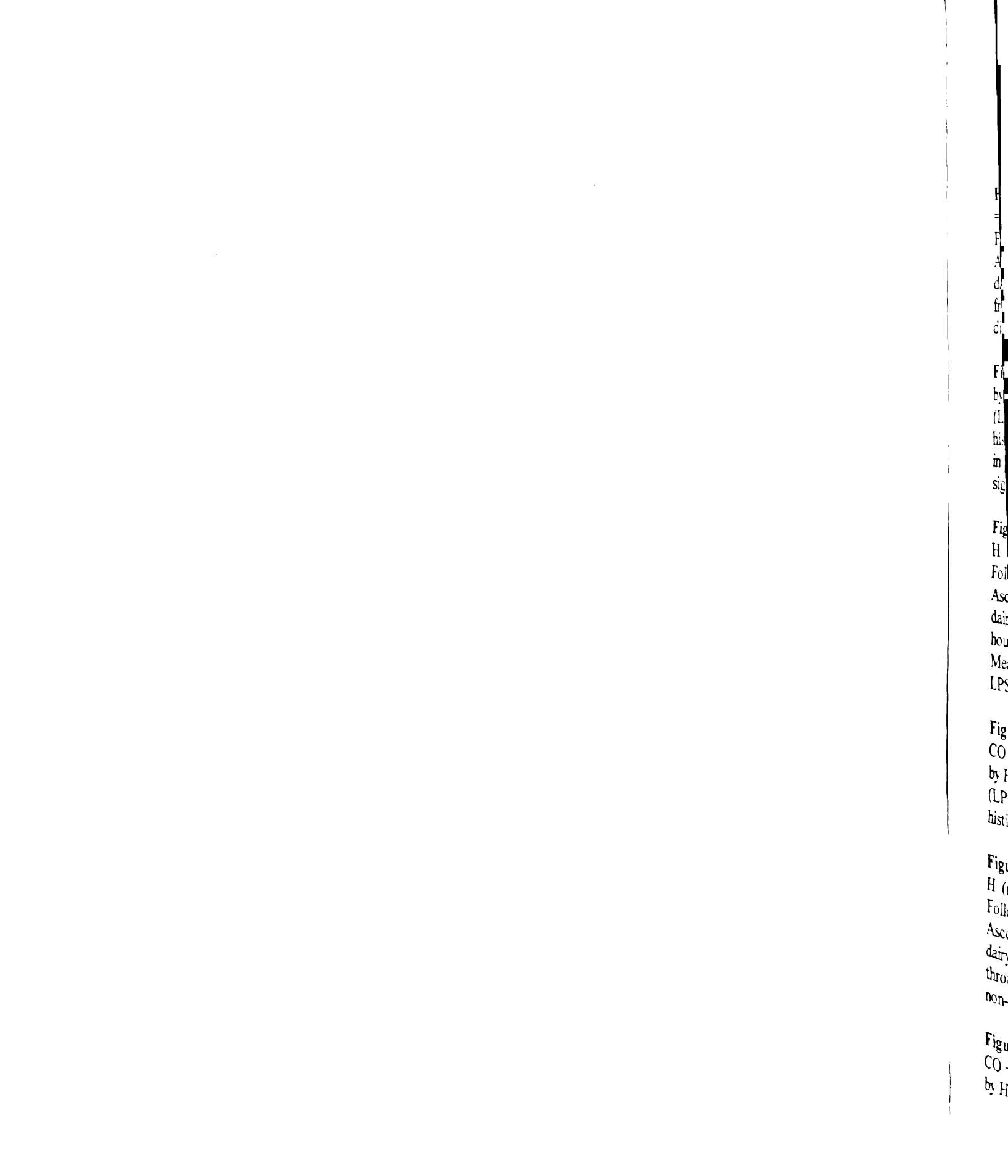

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LIST OF SYMBOLS & ABBREVIATIONS

AA = ascorbic acid

AAPH = 2,2'-azobis (2-amidinopropane) hydrochloride

AAUA = ascorbic acid & uric acid

ADCC = antibody-dependent cell mediated cytotoxicity

AOA = antioxidant activities

B-PE = B-phycoerythrin

BSA = bovine serum albumin

cfu = colony-forming unit

DHIA = Dairy Herd Improvement Association

DNA = deoxyribonucleic acid

DMI = dry matter intake

EDTA = ethylenediaminotetraacetate

ERS = electronic spin resonance

FL = fluorescence

GSH-Px = glutathione peroxidase

H = L-histidine

H_2O_2 = hydrogen peroxide

HPLC = high performance (pressure) liquid chromatography

HR = heart rate

HSCC = high somatic cell count

IgG = immunoglobulin G

IgG_1 = immunoglobulin G_1

IL = interleukin

IMI = intramammary infection

IU = international unit

KLH = keyhole limpet hemocyanin

L-His = L-histidine

LSCC = low somatic cell count

LPS = lipopolysaccharide

LT = leukotriene

LTB4 = leukotriene B4

MHC = major histocompatibility

NSAIDs = non-steroidal anti-inflammatory drugs

$^{\bullet}OH$ = hydroxyl radical

PG = prostaglandin

PGE_2 = prostaglandin E_2

$PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$

PMN(s)= polymorphonuclear neutrophil(s)

PSS = physiological saline solution

RCR = ruminal contraction rate

rpm = round/min

RR = respiratory rate

SAS = Statistical Analysis System

SCC = somatic cell count

SEM = standard error of the means

SRID = Single Radial Immunodiffusion

TMR = total mixed ration(s)

TNF-α = tumor necrosis factor-α

INTRODUCTION

Mastitis, or mammary inflammation, is generally considered to be the most costly disease in dairy cattle throughout the world. Economic losses due to mastitis include decreased production, discarded milk, culling, mortality, labor, veterinary service, medication, and delayed genetic progress (Reneau, 1993). Based on the degree of inflammation, it may be classified as subclinical, subacute clinical, acute, peracute, chronic, and nonbacterial mastitis. Hogan et al. (1989a) gave four guidelines of clinical mastitis cases determined by retrospective reports of clinical signs and culture results of foremilk samples of environmental mastitis reported by Smith et al. (1985). First, a new case of clinical mastitis occurred when a 14-day period had elapsed between reports of clinical signs, regardless of the bacteriological status of the quarter. Second, a new case of clinical mastitis occurred when a different pathogen was isolated from a clinical quarter regardless of the number of days between isolation of dissimilar pathogens. Third, when one or more pairs of duplicate milk samples were cultured during a 14-day period and a pathogen was isolated from one or more pairs of samples, but the remainder of samples were bacteriological negative, the isolated pathogen was determined the cause of the clinical cases. Finally, a new case of clinical mastitis was not recorded if the same pathogen was isolated or if samples were bacteriological negative when less than 14 days had elapsed between reports of clinical signs.

A proper mastitis control program, i.e. post-milking teat dipping, total dry cow therapy, culling, and proper maintenance of milking equipment (Bramley et al., 1984), cannot completely eliminate mastitis from a dairy herd, particularly mastitis caused by

environmental pathogens. Among clinical mastitis cases, coliform organisms are the most common cause of severe cases. The problem of coliform mastitis has not been effectively solved. However, nutrition, especially supplementation of antioxidant micronutrients, is an important part of coliform mastitis prevention because of its critical role in mammary resistance (Bowers, 1997; Erskine, 1993). However, therapeutic potential of antioxidants for the treatment of coliform mastitis has not been investigated. As antioxidants, L-histidine and ascorbic acid have been suggested as potential therapeutics to alleviate free radical-mediated damage in a variety of clinical models.

My hypothesis is that therapy with histidine and/or ascorbic acid will reduce the systemic and local inflammatory response resulting from endotoxin-induced mastitis in dairy cattle. Therefore, this study was conducted with two primary objectives. (1) To determine the effect of parenteral histidine and/or ascorbic acid treatment on acute mammary inflammation. (2) To determine the effect of parenteral histidine and/or ascorbic acid on systemic variables resulting from acute mammary inflammation.

REVIEW OF LITERATURE

Epidemiology of Clinical and Acute Clinical Mastitis.

Dairy herds that have controlled contagious mastitis can still have an unacceptable incidence of intramammary infection (IMI) and clinical cases caused by environmental pathogens (Hogan et al., 1989a; Smith et al., 1985). Procedures, such as post-milking teat dipping, total dry cow therapy, culling, and proper maintenance of milking equipment are successful in reducing the reservoir of contagious pathogens (Bramley et al., 1984). However, they are not generally effective in the control of environmental pathogens (Smith et al., 1985) because, as opposed to contagious pathogens, infections do not generally occur during milking.

The average herd incidence of clinical mastitis in low-somatic-cell-count (LSCC) herds from California, Ohio, and Pennsylvania was 45-50 cases/100 cow-years. And coliforms, lactose-fermenting, gram-negative bacilli of the family *Enterobacteriaceae*, were the predominant pathogen, isolated from 30 to 40% of the clinical cases (Erskine et al., 1988; Gonzales et al., 1990; Hogan et al., 1989a; Smith et al., 1985). In two additional studies from Ohio, 46.5% of microbiological cultures of milk samples from clinical mastitis cases (824/1772) yielded coliform organisms. And *E. coli*, *Klebsiella sp.*, and *Enterobacter sp.* accounted for 14.6%, 2.6%, 2.8% of the clinical cases, respectively (Bartlett et al., 1992). It is suspected that when no bacteria are isolated on culture it frequently results from coliform infections that have been eliminated by the cow's defenses (Bartlett et al., 1993). In the Pennsylvania study, the proportion of clinical mastitis cases

attributable to coliform bacteria was significantly ($P < 0.005$) higher in low somatic cell count (LSCC, ≤ 150,000 cells/ml) herds (43.5 ± 3.5%, n = 12) than in high somatic cell count (HSCC, ≥ 700,000 cells/ml) herds (8.0 ± 3.4%, n = 6) (Erskine et al., 1988). An increased incidence of coliform mastitis is also associated with the first month of lactation, and warm humid weather (Erskine et al., 1988; Hogan et al., 1989a; Smith et al., 1985).

Bedding materials are implicated as primary sources of environmental pathogens during inter-milking periods. The number and type of bacteria in bedding are related to microbial numbers on the teat end (Janzen et al., 1982; Natzke et al., 1976). Hogan et al. (1989b) reported that organic bedding materials, such as sawdust and chopped straw, had significantly higher moisture content and coliform bacteria concentrations ($P < 0.05$) than did sand and crushed limestone. Fundamentally, moisture, available nutrients, and proper temperature are the ecological factors for colonization and multiplication of bacteria. Thus, these factors are critically associated with significantly greater bacterial counts in organic as compared to inorganic bedding materials (Hogan et al., 1989b). The average coliform count in organic materials is significantly higher ($P < 0.05$) during summer (6.5 ± 0.3 colony-forming unit (cfu) $\log_{10}$/g dry weight) than other seasons (5.7 ± 0.4 cfu $\log_{10}$/g dry weight) (Hogan et al., 1989b). The greater coliform counts are probably related to a higher ambient temperature (Hogan et al., 1989b), and coincided with the highest rate of clinical mastitis during summer (Erskine et al., 1988; Hogan et al., 1989a). Additionally, increasing parity is associated with an increased rate of coliform mastitis (Smith et al., 1985).

Hogan et al. (1989a) also found that coliforms accounted for 58.9% (56/95) of

severe clinical cases, and 29.2% (56/192) of clinical coliform cases were classified as severe mastitis with abnormal milk, swelling of quarter, and systemic signs as summarized in a review by Eberhart et al. (1979).

Economic impact.

Severe coliform mastitis causes a tremendous reduction in both milk quantity and quality (Dobbins, 1977; Kitchen, 1981; Schalm, 1977). Losses to dairy producers include decreased production, discarded milk, culling, mortality, labor, veterinary service, medication, and delayed genetic progress (Reneau, 1993). In 50 Ohio dairy herds with a total of 4068 cow-years, the costs per cow-year for clinical cases of mastitis caused by *E. coli* was \$3.21 ± 0.12, which was higher than mastitis caused by other pathogens (Miller et al., 1993).

Pathogenesis.

Polymorphonuclear neutrophil (PMN) phagocytosis is the most critical part of the cow's mammary defense for bacterial clearance (Kehrli, 1994). Four important physiological functions of PMNs are involved in the inflammatory reaction, namely chemotaxis, diapedesis, phagocytosis, and intracellular killing (Burvenich et al., 1994). In response to inflammatory stimuli, PMNs are released from circulating and marginal storage pools, and adhere to blood vessel walls (Burvenich et al., 1994). Then, there is a rapid and massive influx of neutrophils from peripheral blood into the alveolar lumen of the mammary gland, thus markedly increasing the somatic cell count in milk (Burvenich et al., 1994). Lin et al. (1995) supported this concept and demonstrated *in vitro* the process of bovine neutrophil diapedesis across bovine mammary gland epithelial cells (MAC-T).

Light and transmission electron microscopy revealed the sequential neutrophil transmigration, accumulation of neutrophils on the surface of epithelial monolayer, projection of pseudopods into intercellular junctions and movement of neutrophils between adjacent epithelial cells, reapproximation of the lateral epithelial cell membranes, and reformation of the epical tight junctions after neutrophils crossed the bovine mammary gland epithelium (Lin et al., 1995). Following diapedesis, neutropenia or neutrophilia in blood initially occurs with a possible left shift appearance. Neutropenia and depletion of bone marrow reserves of neutrophils follows (Jain et al., 1978). Replenishment of blood and bone marrow neutrophil pools from compensatory stimulation of granulopoiesis is often associated with subsidence of acute mastitis and recovery (Jain et al., 1978). The speed at which neutrophils are mobilized into the gland is a primary determinant of the severity of coliform mastitis cases during lactation (Hill, 1981).

The emigration of leukocytes, particularly PMNs and monocytes, out of the blood vessels takes place independently of the increased vascular permeability of acute inflammation (Tizard, 1996). This process, known as an extravasation, depends on adhesive interactions activated by the local release of inflammatory mediators (Janeway et al., 1997; Tizard, 1996). Binding results when endothelial cells express adherence molecules. This expression is triggered by bacterial components (e.g. lipopolysaccharide, LPS) or inflammatory mediators, i.e. thrombin, histamine, tumor necrosis factor [TNF-α] and interleukin-1 (IL-1) (Janeway et al., 1997; Tizard, 1996). Adhesive glycoprotein molecules namely P-selectin (CD62P) and E-selectin (CD62E) mediate the first step of the process (Janeway et al., 1997). P-selectin, which is normally stored in granules (Weibel-

Palade bodies) in endothelial cells, is translocated to the endothelial cell surfaces within a few minutes of exposure to Leukotriene-B4 (LTB4), C5a, or histamine (Janeway et al., 1997). E-selectin appears a few hours after exposure to LPS or TNF-α. These selectins can bind to carbohydrate side chains, sialyl-Lewis^x moiety (s-Le^x) on neutrophil surface glycoproteins (Janeway et al., 1997). The adhesion in this step is weak and allows leukocytes to roll along the vascular endothelial surface (Janeway et al., 1997; Tizard, 1996). This adhesive interaction enhances the stronger interactions of the second step, which depends upon the leukocyte integrins, LFA-1 (CD11a:CD18), and the immunoglobulin-related molecule ICAM-1 on endothelial surfaces (Janeway et al., 1997; Tizard, 1996). Platelet-activating factor (PAF) secreted by endothelial cells activates the rolling neutrophils (Janeway et al., 1997; Tizard, 1996). Then, LFA-1 is increasingly expressed on the neutrophil surface, which results in an increased affinity for ICAM-2 (Janeway et al., 1997; Tizard, 1996). IL-8 produced from endothelial cells by the induction of IL-1 triggers a conformational change in LFA-1, which increases its adhesive capacity. Subsequently, neutrophils adhere firmly to the endothelium (Janeway et al., 1997; Tizard, 1996). IL-8 also acts as a chemotactic molecule to attract more PMNs to the area (Tizard, 1996). In the third step, CD31, an immunoglobulin-related molecule, is expressed on leukocytes and at the intercellular junction of endothelial cells. This enhances neutrophils to penetrate across the endothelium. The last step is under the influence of cytokines, thereafter, phagocytosis take places (Janeway et al., 1997; Tizard, 1996).

In phagocytosis, PMNs primary granules fuse with the phagosomes to form

phagolysosomes. Since this fusion may occur before the pathogen or LPS is completely ingested, the lysosomal contents may be released into the mammary tissues (Janeway et al., 1997; Tizard, 1996). After the ingestion of pathogens, phagocytes will increase their oxygen consumption 10 times as much as that of resting cells. This cellular oxidative mechanism of the PMN termed "the respiratory burst" (Chew, 1996; DeChatelet, 1978) generates potent oxidizing agents also called oxygen-derived radicals, molecule with an odd number of electrons (VanSteenhouse, 1987). They include singlet oxygen ($^1O_2$), peroxides (H_2O_2), and free hydroxyl radicals ($^{\bullet}OH$). H_2O_2 is included because its potential for the rapid production of $^{\bullet}OH$ in the presence of an iron catalyst via the Fenton reaction in equation III (VanSteenhouse, 1987).

(Haber-Weiss) $O_2^- + H_2O_2 \longrightarrow O_2 + {}^{\bullet}OH + {}^-OH$ .....(Equation I)

$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$.....(Equation II)

(Fenton) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + {}^-OH$.....(Equation III)

These agents destroy the invading microbes or their products (e.g., LPS). Concomitantly, they also provide the harmful activities associated with oxidative damage to host cell membrane, enzymes and nucleotides in DNA (Bendich, 1993; Chew, 1996; Machlin & Bendich, 1987; VanSteenhouse, 1987).

PMNs isolated from mammary secretion are less efficient than PMNs isolated from peripheral blood (Pappe et al., 1977). Their decreased phagocytic and bactericidal activities had been associated with many factors—decreased intracellular glycogen reserves, ingested milk fat globules and casein, (Pappe et al., 1977) inadequate level of opsonins, and cortisol levels (Fox et al., 1981).

Endotoxin also called lipopolysaccharide is a virulent factor and a cell wall component of gram-negative bacteria (Raetz, 1993). It is released from the gram-negative bacteria upon cell death and composed of three basic subunits; O-specific polysaccharide, Lipid A, and R-core (Raetz, 1993). Although endotoxin itself has no direct damaging effect on mammary epithelium (Frost, 1984), it can cause pathophysiological effects, which are predominantly dose-dependent (Giri, et al., 1984; Lohuis et al., 1988b). Generally, as dosage increases latency time decreases, the peak effect becomes more pronounced, and the duration of the effect protracted (Lohuis et al., 1988b). It also induces host inflammatory mediators (Shuster et al., 1993). When mammary tissues are stimulated, phospholipases act on the phospholipids in cell wall to release fatty acids including the most important unsaturated long-chain fatty acid, arachidonic acid (Tizard, 1996). Two enzymes, including 5-lipoxygenase and cyclooxygenase result in arachidonic acid metabolites (Tizard, 1996). Under the influence of the former, arachidonic acid is converted to biologically active lipids called leukotrienes (LT). While under the influence of the latter, arachidonic acid yields prostaglandin (PG) series, i.e., PGA_2 (Thromboxane-A_2), PGE_2, $PGF_{2\alpha}$, PGI_2 (Prostacyclin) (Tizard, 1996). Other inflammatory mediators include cytokines, interleukin (IL-1, IL-6, IL-8) and tumor necrosis factor-α [TNF-α], and complements, such as C5a as chemotactic factors, and vasoactive factors such as histamine (Tizard, 1996). The mechanism results in local inflammation with five cardinal signs, i.e. redness, swelling, pain, heat, and disturbed mammary function within a few hours after intramammary infusion of endotoxin (Lohuis et al., 1988b; Tizard, 1996). However, a study in non-pregnant, lactating cows demonstrated that the intravenous

administration of 100 μg of LPS did not induce clinical mastitis (Shuster et al., 1991c). The subsequent absorption of endotoxin-induced inflammatory endogenous mediators in the udder rather than absorption of endotoxin itself into the circulation (Lohuis et al., 1988) causes systemic signs, i.e., fever, acute phase reactants, metabolic changes, and vascular responses of the host (Lohuis et al., 1988b; Tizard, 1996).

Endotoxin by either intracisternal or intravenous route causes pathophysiological effects on lactational performance by suppressing milk yield in affected quarters as well as unaffected ones (Shuster et al., 1991a, 1991b, 1991c). However, a more severe and prolonged suppression occurred in infused quarters compared to uninfused ones, which are consequently affected by systemic responses (Shuster et al., 1991a).

Intramammary infusion of endotoxin does not result in as markedly decreased rumen motility in contrast to the intravenous route (Lohuis et al., 1988), or experimental and natural *E. coli* mastitis (Verheijden et al., 1983). However, clinicopathological changes including expanded plasma volume, hyponatremia, transient hyperchloremia and hypophosphatemia, hypocalcemia, and decreased serum activities of liver- and muscle-specific enzymes, have been well demonstrated (Tyler et al., 1994a).

Acute Phase Response.

The systemic events that result from acute endotoxin-induced mastitis are collectively termed the acute phase response (Bishop et al., 1976). These include fever, increased serum cortisol, increased serum concentrations of proteins (fibrinogen, complement, haptoglobin, and ceruloplasmin), transient decreases of serum Fe and Zn, and mobilization of leukocytes. These events have been demonstrated in cows with acute mastitis (Conner et al., 1986; Erskine et al., 1989; Erskine et al., 1993; Jackson *et al.*,

1990; Lohuis et al., 1990; Shuster et al., 1992). In cows experimentally administered *E. coli* endotoxin, the average serum cortisol peaked significantly ($P < 0.05$) higher (100 vs. 82 ng/ml) and sooner (2.5 vs. 4.5 hr posttreatment) in intravenous (n = 4) as compared to the intramammary (n = 12) treatment group (Jackson et al., 1990). In cows intracisternally challenged with 50 colony-forming units (cfu) of *E. coli,* mean serum concentrations of Zn and Fe decreased 21-24% and 28-35%, respectively (Erskine & Bartlett, 1993; Lohuis et al., 1988), and mean serum concentration of Cu decreased to 52% of prechallenge concentrations (Erskine & Bartlett, 1993). Three plasma proteins; haptoglobin, ceruloplasmin, and α_1-antitrypsin classified as acute phase reactants were higher in the cows with mastitis than non-affected cows (Conner et al., 1986; Tizard, 1996). In particular, the iron-binding protein haptoglobin is considered a major acute-phase protein in ruminants (Alsemgeest et al., 1994; Tizard, 1996). A 52-fold increase in serum haptoglobin was detected by a high performance liquid chromatography (HPLC) method from serum samples taken from eight cows with experimentally *E. coli*-induced mastitis. (Salonen et al., 1996).

Politis et al. (1991) found that various concentrations (0-30 μg/ml) of *E. coli* LPS did not affect the expression of major histocompatibility (MHC) class II molecules on the surface of bovine mammary macrophages *in vitro*. In addition, LPS was unable to enhance the proliferation of antigen-specific T-cells (Politis et al., 1991).

Therapy & Prevention.

Anderson (1989) suggested the therapeutic management of acute coliform mastitis should be based on early, accurate detection and careful clinical assessment. Therapeutic

principles identified for management of acute coliform mastitis include elimination of bacteria from the mammary gland, neutralizing the effects of endotoxin, and providing supportive therapy (Anderson, 1987). Antimicrobial agents, particularly extra-labeled drugs, have been promoted as the primary regimen for bacterial elimination. Antimicrobial agents alone, however, have minimal benefit in the treatment of clinical gram-negative mastitis (Erskine et al., 1992; Jones et al., 1990). Most cases spontaneously recover without antimicrobial therapy (Anderson, 1989). Additionally, milk discard costs to avoid drug residues in marketed milk are potentially the most costly consequence of antibiotic use (Erskine et al., 1991, 1992). Thus, antimicrobial treatment should only be considered as an adjunct to other supportive care to alleviate the effects of shock (Erskine et al., 1991). This includes anti-inflammatory treatment (Anderson et al., 1986; DeGraves et al., 1993; Lohuis et al., 1988a, 1989), and administration of calcium, glucose, and hypertonic saline solution (Anderson, 1989; Constable et al., 1991; Cullor, 1993; Tyler et al., 1994b). Anti-inflammatory treatment of coliform mastitis with either steroids (Lohuis et al., 1989); dexamethasone (Lohuis et al., 1988a) or non-steroidal anti-inflammatory drugs (NSAIDs) such as flunixin meglumine (Anderson et al., 1986), and ibuprofen (DeGraves et al., 1993) enhances clinical outcomes of experimental coliform mastitis.

Endotoxin-induced shock is complex involving cardiogenic, hypovolemic, neurogenic and other mechanisms (Constable et al., 1991; Smith, 1986). The technique of hypertonic saline infusion has proved to be a useful adjunct in treatment of the outcome of those mechanisms (Erskine et al., 1994; Sargison et al., 1996; Tyler et al., 1994). Intravenous administration of 5ml/kg of hypertonic saline solution (7.2-7.5% NaCl)

increased plasma volume in cows with endotoxin-induced mastitis and endotoxin induced shock compared to cows that were administered isotonic NaCl solution (Erskine et al., 1994; Sargison et al., 1996; Tyler et al., 1994). Mechanisms of action of hypertonic saline may include redistribution of body water, which enhances circulatory blood volume and tissue perfusion, a vagal-mediated ionotropic effect on the heart, and altered peripheral vascular resistance or a combination of these factors (Sargison et al., 1996).

Effective and economic coliform mastitis control programs rely on prevention rather than treatment (Erskine et al., 1993); therefore, milking hygiene, teat dipping, and environmental sanitation should be major objectives (Anderson, 1989). Additionally, vaccination programs, including *E. coli* J5 vaccine can be helpful. The *E. coli* J5 vaccine is a bacterin produced from a mutation of *E. coli* O111:B4 strain J5 (Rc mutant), which lacks the "O" antigen capsular portion of the cell wall (Cullor, 1991; Gonzalez et al, 1989). This mutant thus has the core antigen (LPS) portion of the cell wall exposed to possible immune recognition (Cullor, 1991; Gonzalez et al, 1989). Using the core antigen as an immunogen reduces the requirement for antibody diversity. This is important because coliform mastitis infections are caused by numerous serotypes of gram-negative bacteria (Fang & Pyorala, 1996; Gonzalez et al., 1989; Tyler et al., 1990). The severity of clinical signs in experimental infections (Hogan et al., 1992b), and the incidence of clinical cases of coliform mastitis during the first three month of lactation have been decreased through vaccination (Gonzalez et al., 1989; Hogan et al., 1992a). *Escherichia coli* J5 vaccination should be profitable when incidence of coliform mastitis exceeds 1% (DeGraves et al., 1991).

Nutrition in Coliform Mastitis.

Nutrition, particularly supplementation of antioxidant micronutrients, plays a critical role in mammary resistance and phagocytic function (Bowers, 1997; Erskine, 1993). The role of antioxidant vitamins including vitamin A, vitamin E, ascorbic acid and β-carotene has been studied as well as minerals—Selenium (Se), Zinc (Zn), Copper (Cu), and Iron (Fe) (Chew, 1996; Erskine, 1993). In particular, studies have demonstrated the role of vitamin E and Se in host resistance to coliform mastitis. Grasso et al. (1990) demonstrated that dietary Se supplementation in cows increased bovine PMN phagocytosis and killing, and decreased extracellular hydrogen peroxide (H_2O_2) production. Experimentally induced intramammary *E. coli* (15-40 cfu) infections were significantly ($P < 0.05$) more severe, and of longer duration (114.4 ± 18.0 hr) in Se-deficient Holstein cows (162.0 ± 12.0 hr, n = 10) than in a Selenium-supplemented group (Erskine *et. al.*, 1989). Supplementation of Se and vitamin E during the dry period decreased (62%) the duration of clinical mastitis, while the incidence of clinical mastitis was reduced (37%) by vitamin E (740 IU/d) alone (Smith et al., 1984). Selenium and vitamin E are associated with lower milk SCC (Erskine et al., 1987). Selenium is required for glutathione-peroxidase (GSH-Px) activities (Bendich, 1993). The whole blood concentrations of Se and GSH-Px activity were significantly higher ($P < 0.01$) in low SCC dairy herds (n = 16, 0.133 ± 0.01 μg/ml and 35.6 ± 2.95 mU/mg of Hb) than in high SCC herds (n = 16, 0.074 ± 0.007 μg/ml and 20.2 ± 2.38 mU/mg of Hb) (Erskine et al., 1987).

Antioxidants as Therapy.

Therapeutic modulation of the local inflammatory and systemic response of clinical

coliform mastitis is not fully understood. Fundamentally, cellular mechanisms of host defense should not be totally obstructed by therapy. The role of antioxidant vitamins and minerals as part of a therapeutic regimen has not been studied. Studies of single nutrients may be misleading because interactions are not considered; therefore, more research on the effects of multiple nutrients is needed (Jacob, 1995).

Ascorbic Acid & L-histidine.

Ascorbic acid

Ascorbic acid is produced by the liver of many animals including cattle (Eicher-Pruiett, et al., 1992; Itze, 1984). Hence, the biosynthetic capacity for ascorbic acid in adult ruminants is sufficient to cover the ascorbic acid requirement (Itze, 1984). Nonetheless, ruminants can be prone to ascorbic deficiency due to an impaired synthesis, and a rapid destruction by the ruminal microflora via oral administration of ascorbic acid (Itze, 1984). Because of the irritation following intramuscular and subcutaneous injection, the best means of administration of ascorbic acid is by intravenous injection (Loscher et al., 1984).

Ascorbic acid is a water-soluble cytosolic chain-breaking antioxidant (Machlin & Bendich, 1987). It quenches free radicals as well as singlet oxygen (Bodannes et al., 1979; Machlin & Bendich, 1987; Niki, 1991b) by providing hydrogen atoms to pair up with unpaired electrons on free radicals in the aqueous compartments such as blood plasma and cell cytosol (Jacob, 1995). Dwenger et al. (1994) suggested that scavenging of reactive oxygen metabolites by ascorbic acid is responsible for the improvement of endotoxin-induced acute lung injury. *In vitro*, chemiluminescence response of following

zymosan exposure was significantly higher in PMNs collected from sheep treated with endotoxin (0.5 μg/kg body weight, i.v.; *E. coli* endotoxin O55:B5) than in PMNs from sheep treated with endotoxin endotoxin and ascorbic acid group (0.5 μg/kg body weight, *E. coli* endotoxin O55:B5 & 1 g/kg body weight, i.v. bolus injection followed by 0.2 g/kg per hr continuous infusion of ascorbic acid) (Dwenger et al., 1994).

The mortality rate from bacterial septicemia in channel catfish decreased with increased dietary ascorbic acid from 100% (0 mg/kg) to 15% (300mg/kg) and 0% (3000 mg/kg) (Li et al., 1985). In chickens, 330 mg of ascorbic acid/ kg of feed reduced mortality and pericarditis (46/60, 76%) after challenging with *Escherichia coli* (O1:K1) in air sacs compared to unsupplemented controls (12/63, 19%) (Gross et al., 1988). In cattle, given 20 mg/kg body weight ascorbic acid subcutaneously, (n = 15) neutrophil oxidative metabolism and capability of neutrophils to mediate antibody-dependent cell mediated cytotoxicity (ADCC) were enhanced ($P < 0.05$) (Roth et al., 1985). Conversely, in young calves, ascorbic acid appeared to have beneficial as well as adverse effects (Eicher-Pruiett et al., 1992). Young calves (n = 10) supplemented orally with 10 g of ascorbic acid had reduced ocular and nasal discharge ($P < 0.01$), but had more fluid feces and impaired neutrophil function (neutrophil-mediated phagocytosis and antibody-dependent cellular cytotoxicity) (Eicher-Pruiett et al., 1992). Cummins and Brunner (1989) determined that ascorbic acid (1.75 g/d) decreased plasma IgG concentrations and plasma antibody titers to a specific antigen (keyhole limpet hemocyanin, KLH) in young calves (n = 6), but also decreased the incidence of scouring.

Ascorbic acid can also synergistically interact with other antioxidants. Ascorbic

acid synergistically restores radical scavenging activity of vitamin E (Machlin & Bendich, 1987; Niki, 1991b), and protects cell membrane against peroxidation (Eicher-Pruiett et al., 1992; Niki, 1991b). Ascorbic acid interacts with the tocopheroxyl radical in order to regenerate tocopherol, the active form of vitamin E (Jacob, 1995; Machlin et al.,1987). In young calves, the adverse effect of ascorbic acid supplementation on neutrophil functions was negated by simultaneously feeding of 57 IU/kg of vitamin E in dry milk replacer (Eicher-Pruiett et al., 1992).

L-histidine

L-histidine, an essential amino acid (Chalupa & Sniffen, 1991; Peterson et al., 1998), has been classified as an antioxidant (Kawamoto et al., 1997; Peterson et al., 1998). Evidence supported L-His as an extremely effective scavenger of $^{\bullet}OH$ by decreasing electron spin resonance (ESR) signal intensity of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO)-OH spin adduction in electron-spin-resonance spectroscopy (Nagy & Floyd, 1984). In mice, L-His reduced intestinal membrane permeability in a model experimental bacterial diarrhea (Peterson et al., 1998). The mean fluid-accumulation response in intraperitoneally L-His-treated mice (100 μl of 238 mM, n = 22) challenged with *Salmonella typhimurium* was 76±14 μl/cm, which was significantly lower (47%, P = 0.0002) than that of the *S. typhimurium*-challenged control mice (143 ± 10 μl/cm, n = 28) (Peterson et al., 1998). Kawamoto et al. (1997) reported L-His protected against ischemic/reperfusion-induced injury in the cerebrum of the rat. Intravenous administration of 50 mg/kg and 100 mg/kg L-His delayed neuronal death and maintained the neuronal density of the forebrain in rat hippocampus ($P < 0.01$) (Kawamoto et al., 1997). Because

of a short half-life, L-His is rapidly metabolized and/or excreted (45 min in mice and 1.7 hr in human) (Peterson et al., 1998; Sitton et al., 1988).

L-histidine serves as a precursor of histamine, which is synthesized locally by the enzyme histidine decarboxylase in mast cells (Babizhayev et al., 1994; Maslinski et al, 1993), but not in enterocytes (Guihot & Blachier, 1997). Maslinski et al. (1993) found that histamine concentrations in bovine milk were higher (317 ± 29 nmol/l, n = 6) than that in bovine plasma (4.83 ± 0.82 nmol/l, n = 5). Histamine concentration in bovine milk was higher (600 nmol/g) than in other mammals' milk (guinea pig, mouse, rat, and pig) (Maslinski et al, 1993). Histamine affects blood vessels, smooth muscle and exocrine glands (Tizard, 1996), and is believed to contract myoepithelial cells of alveoli and small ducts in mammary gland, that in turn stimulate milk secretion or milk ejection (Maslinski et al, 1993). Hence, L-His may indirectly elicit these responses through histamine induction during clinical mastitis.

Although studies demonstrating the potential benefits of ascorbic acid and L-His in various laboratory animals are well recognized (Bushell et al., 1996; Cummins and Brunner, 1989; Eicher-Pruiett et al., 1992; Gross et al., 1988; Kawamoto et al., 1997; Li et al., 1985; Maslinski et al, 1993; Peterson et al., 1998; Roth et al., 1985). Potential benefits in cows with coliform mastitis are unknown. As antioxidants, they may ameliorate clinical changes caused by shock, and perhaps shock caused by endotoxin-induced mastitis.

MATERIALS AND METHODS

I. Cows

We selected eight, non-pregnant Holstein cows with clinically normal milk and mammary glands, quarter somatic cell counts less than 500,000 cells/ml and negative bacterial cultures at 24 hr before endotoxin challenge. Cows were fed a total mixed ration (TMR) balanced for 90-lb milk production and housed in tie stalls. Data regarding age, milk production, day of lactation, and lactation number was recorded. A jugular catheter was aseptically inserted at 12 hr before endotoxin challenge and remained in each cow until the end of data collection.

II. Endotoxin-induced mastitis

Endotoxin solution (20 μg/ml) was prepared by dissolving 100 μg of a commercial (Sigma) *Escherichia coli* O111:B4 endotoxin in 5 ml of pyrogen-free physiological saline solution (PSS), which was then filtered by a 0.22-μm low extractable filter unit (Sterile® D-GS, Millipore Industria E. Comerico Ltda.). The suspension was stored at 4 °C and vigorously shaken before infusion. On Tuesday mornings, soon after milking, the entire 100-μg preparation was intracisternally infused into 1 quarter/cow via syringe and 1 1/3" disposable J-12 teat infusion cannula. Before infusion, the teat was aseptically prepared with alcohol. The infused teats and quarters were immediately massaged for 15-20 seconds in order to distribute endotoxin.

III. L-histidine & Ascorbic Acid Solution Preparation.

L-histidine (ICN Biomedicals Inc.) solution was prepared by dissolving 25 g of L-

His in 500 ml of pyrogen-free PSS and dissolving with a stir bar on a warm magnetic stirrer for approximately 35 min. The solution was then filtered with a 0.22-μm low extractable filter unit (Sterile® D-GS, Millipore Industria E. Comerico Ltda.). Ascorbic acid solution was also prepared from ascorbic acid injectable solution (The Butler Company) by diluting 25 g of ascorbic acid into in 500 ml of pyrogen-free PSS. The solution was vortexed and then filtered with a 0.22-μm low extractable filter unit (Sterile® D-GS, Millipore Industria E. Comerico Ltda.).

Two 25-g doses of L-His and/or ascorbic acid were slowly administered by intravenous injection via the jugular catheter. In order to mimic a clinical case of coliform mastitis, the first dose was administered intravenously at 3 hr after endotoxin challenge to allow time for clinical signs to appear. Thereafter, the second dose of 25 g was administered at 5-hr post endotoxin challenge.

IV. Experimental design

The Latin square cross-over design (4 x 4 table) was used in diffferent orders. Each Holstein cow was randomly selected to complete each of the four treatments. The treatments included LPS challenge as control (OO), LPS and ascorbic acid (CO), LPS & L-His (OH), and LPS, L-His & ascorbic acid (CH) (Table 29, Appendix B). The experiment was started by using the left-front (LF) quarter of the first cow. The other cows were then randomly assigned to treatment by selecting cow numbers by drawing from a box and proceeding in order down the table (Table 29, Appendix B). Each quarter was used one time for endotoxin-induced mastitis, thus all four quarters were used over the four different periods (each of four successive weeks).

V. Milk Collection

Milk samples were collected at 12 hr before challenge, immediately before challenge, and 2, 3, 4, 6, 9, 12, 24, 36, 48, 60, 72, 96 hr and 1 week after challenge (Table 30, Appendix B). After aseptic preparation and discarding foremilk, milk samples were collected to determine somatic cell count, bacteriology, and immunoglobulin G_1 (IgG_1) concentration. All milk samples were stored in crushed ice immediately after collection. We collected one vial for somatic cell count preserved with a bronopol pellet. This was sent to the DHIA laboratory of Michigan. A second vial was collected for bacteriological culture on 5% sheep blood agar, and incubated for 24 hr at 37 °C. A third sample was collected into a vial with 0.05 ml of 1 M Benzamidine HCl as a protease inhibitor and then centrifuged at 1000 × g for 15 min to separate cells and fat. Whey was prepared for IgG_1 measurement by modifying Guidry's procedure (1980). The skim layer beneath the fat was transferred to a new vial, and 5 µl of glacial acetic acid added to precipitate casein. The solution was then centrifuged at 13,000 rpm (Biofuge pico) for 13 min. The supernatant was decanted into another clean cryovial, 5 µl of KOH was added, and frozen at -20 °C. Commercial IgG_1 Single Radial Immunodiffusion (SRID) kits (VMRD, Inc.) were used to determine IgG_1 concentration.

VI. Blood Collection

Blood samples were collected at 12 hr before challenge, immediately before challenge, and 2, 3, 4, 6, 9, 12, 24, 36, 48, and 72 hr after challenge (Table 30, Appendix B). Blood samples were obtained from a jugular catheter into heparinized vacutainers, and immediately placed in crushed ice. Sodium citrate was used at each blood sampling to

insure anticoagulation in the catheter. Blood samples were centrifuged at 3,000 × g, 4 °C for 15 min to separate plasma. Duplicate 200-µl and 750-µl samples of plasma were pipetted into cryovials for ascorbic acid and antioxidant capacities, respectively. Nitrogen gas was added and samples were stored at -80 °C. Ascorbic acid analysis was performed at the Animal Health Diagnostic Laboratory, Nutrition Section, Michigan State University, Michigan. Antioxidant capacities were analyzed at the laboratory of Dr. R.J. Harmon, University of Kentucky, Lexington, Kentucky.

VII. Ascorbic Acid (AA) Protocol

Plasma ascorbic acid was measured by HPLC using isocratic mobile phase buffers and a reverse-phase, C18 column coupled with electrochemical detection and compared with a known ascorbic acid standard (AAUA-1010). The protocol is described in detail (Appendix D). Briefly, each sample (200 µl) was mixed with buffer (400 µl of 1 mM 90% methanol in water saturated with EDTA) to precipitate protein. They were vortexed, incubated on ice for 10 min, and centrifuged at 3000 rpm, 4 °C for 15 min. The supernatants were then transferred to another set of plastic microtubes and placed on ice. Before running the samples, the column was prepared by passing the mobile phase solution through the entire system and rinsing the pump seal with different concentrations of MeOH. Ten-microliter samples were injected into the prepared HPLC column, and quantified by single-point calibration against a known ascorbic acid standard (AAUA-1010). Peak height or area integration was considered as the response factor.

The content of ascorbic acid was calculated automatically on a Millenium spreadsheet. The internal and external standard value were used in the quantification and determined by the

specific peak of each sample and AAUA, respectively. Both inter- and intra-assay coefficient of variations, which were 13.6% and 5.9%, respectively, were also included in the calculations. The content for each sample was divided by injection volume (10 μl), multiplied by a dilution factor (3, the addition of tissue buffer), and finally reported in mM concentrations of ascorbic acid.

VIII. Antioxidant Activities (AOA) Protocol

The assay measured the antioxidant ability of plasma, which inhibited chemical damage to phycoerythrin induced by the oxidative agent (i.e., mM 2,2'-azobis (2-amidinopropane) hydrochloride, AAPH) and was detected by the rate of phycoerythrin fluorescence emission. It was previously performed (Glazer, 1988), and described in detail for this experiment (Appendix E). Briefly, as a control, the 4-ml final reaction mixtures contained 3.58 ml of 75 mM sodium phosphate buffer (pH 7.0), 0.02 ml of 1.7×10^{-6} M B-phycoerythrin (B-PE), and 0.4 ml of 40 mM 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH, an initiator of the oxidative reaction) at 37 °C. Into each sample tube, 0.2 ml diluted plasma (1:320 dilution in the final volume) was added in the same mixtures in place of 0.2-ml buffer. The solution was excited at 525 nm, and emission was read at 575 nm. The fluorescence was measured at 37 °C in a digital fluorometer immediately before and at 5-minute intervals for 40 min after the addition of AAPH. Sample AOA was calculated and reported as percentage inhibition values of the decay of fluorescence (FL) of the compound phycoerythrin. The percentage inhibition was calculated as:

$$\% = [(\text{change FL control} - \text{change FL sample}) / (\text{change FL control})] \times 100$$

The change in FL was that which occurred over the 40-minute incubation. The greater the

% inhibition, the greater the antioxidant capacity of the plasma. The control in the assay is the rate of decay of fluorescence with no antioxidant present. Hydroxyl radicals are generated by this reaction and cause the decay. The samples were measured in duplicate and the means are displayed on graphs.

IX. Clinical Monitoring

Cows were clinically monitored at 12 hr before challenge, immediately before challenge, and 2, 3, 4, 6, 9, 12, 24, 36, and 48 hr after challenge (Table 30, Appendix B). Rectal temperature, ruminal contraction rate, heart rate, and respiratory rate were all concomitantly measured. Quarter and milk appearance were also observed compared with the appearance before LPS challenge.

X. Milking Procedure

Cows were milked twice daily at approximately 10-12-hour intervals (A.M.-P.M.) by a quarter milking machine from Monday evening through Saturday morning. All quarters were post-dipped with a post-dipping solution soon after each milking. Dry matter intakes (DMIs) were also recorded each day.

XI. Statistical Analysis

A repeated measurement analysis (Statistical Analysis System, SAS® Institute 1989-1996), was used for comparisons among the four treatments (OO, CO, OH, CH). Specific contrasts were used to determine the effects of ascorbic acid, histidine, non-ascorbic acid, and non-histidine group. Period (week 1, 2, 3, & 4), front or hind quarter, and cow were also included as independent variables. The test for sphericity on the GLM printout (Mauchly's criterion) applied to Orthoganol components was used to indicate if a

multivariate analysis was needed. With this statistical method, the following dependent variables were tested: milk production, rectal temperature, log SCC, milk IgG_1, AOA, Heart rate, respiratory rate, and ruminal contraction rate. A period variable was included to adjust for carryover effects of the previous endotoxin or treatments sufficiently long time was not allowed between the administration of different treatments to the same cow. Due to a small sample size (8 cows) with many repeated measure, repeated measures were grouped into 3-4 groups in order of the time. A copy of the SAS and output is shown in Appendix F.

RESULTS

The data was analyzed to compare cows that were administered ascorbic acid (AA) with cows not treated with ascorbic acid, and to compare cows treated with L-histidine (H) with cows not treated with L-His. Comparisons among individual treatment groups (control, ascorbic acid only, L-His only, and ascorbic acid + L-His) were made, however in order to present a concise discussion on the critical hypothesis of this research, the comparisons among treatments are attached in Appendix C.

I. Rectal Temperature

Mean rectal temperature in AA treated cows was significantly lower than in non-AA cows from 24 to 48 hr after LPS challenge ($P = 0.0393$, Figure 1). However, there was no difference between H and non-H cows over the experimental period (Figure 1). Mean rectal temperature in period 1 was significantly lower than in period 2 and 3 from 0 to 4 hr post LPS challenge ($P < 0.04$, Figure 2). Mean rectal temperature in period 4 was also significantly lower than in period 2 and 3 from 3 to 4 hr post LPS challenge ($P < 0.056$, Figure 2). The data is plotted in Figures 1 and 2.

II. Somatic Cell Count

Mean somatic cell count in non-AA cows was significantly higher ($P = 0.0261$, Table 4) than in AA cows at 24 and 36 hr post LPS challenge. Moreover, mean SCC in H cows was significantly lower than in non-H cows from 6 to 24-hr after challenge ($P = 0.0164$). Log SCC looked consistent among treatments (Figure 3). The data is plotted in Figures 2 and 3.

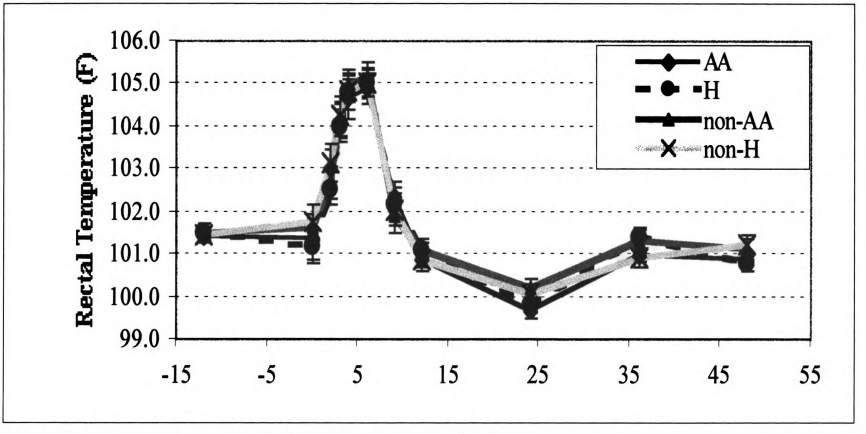

Figure 1 **Mean Rectal Temperature as Treatment Groups; AA (n = 16): CO +** CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. Mean rectal temperature AA cows is significantly lower than in non-AA cows from 24 to 48 hours after LPS challenge (P = 0.0393). However, there were no differences between H and non-H cows all over the experimental period.

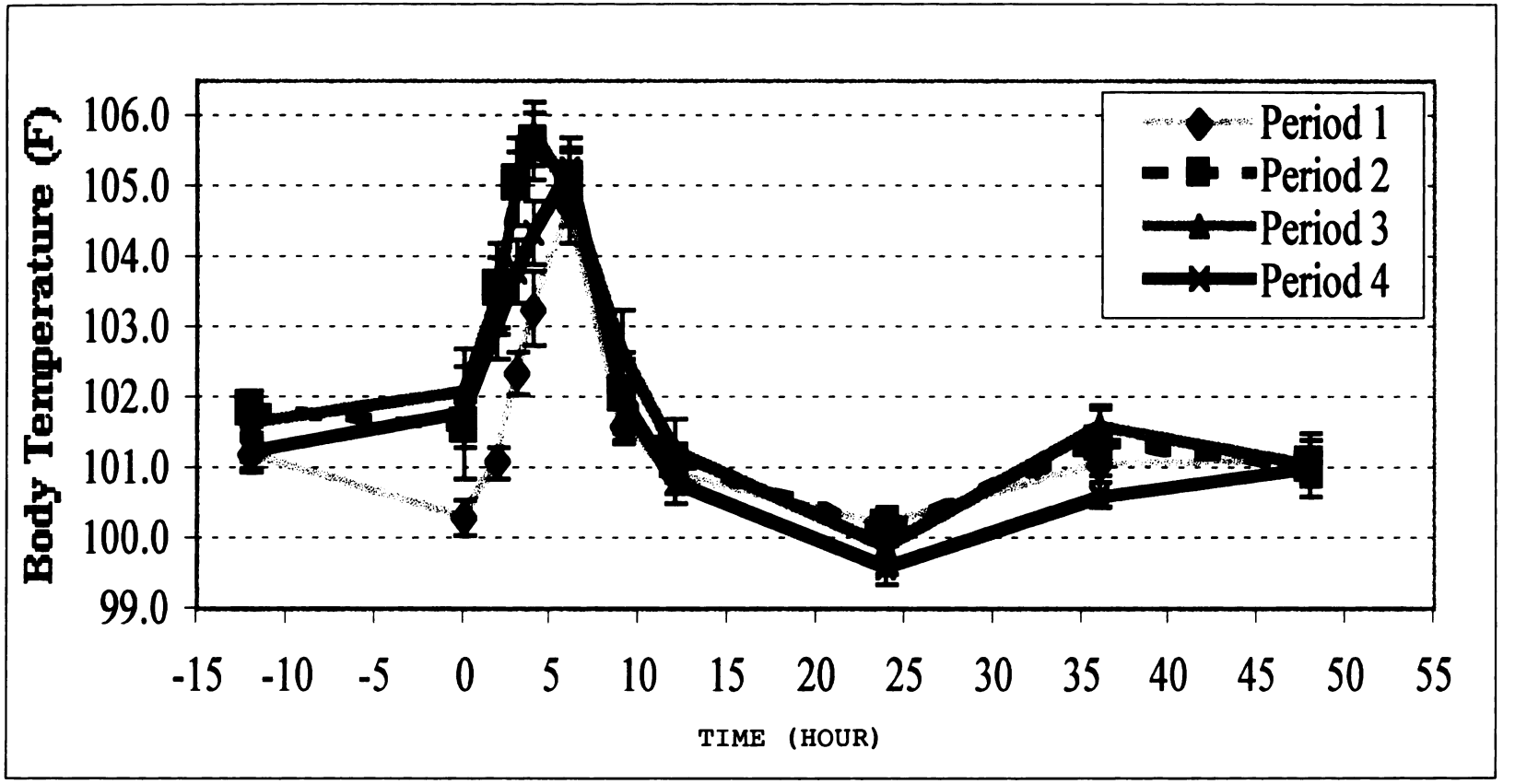

Figure 2 Mean Rectal Temperature as Period Groups; Period 1, 2, 3, & 4 (n = 16 each), by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. Mean rectal temperature in period 1 was significantly lower than in period 2 and 3 from 0 to 4 hr ($P < 0.04$). Mean rectal temperature in period 4 was also significantly lower than in period 2 and 3 from 3 to 4 hr ($P < 0.056$).

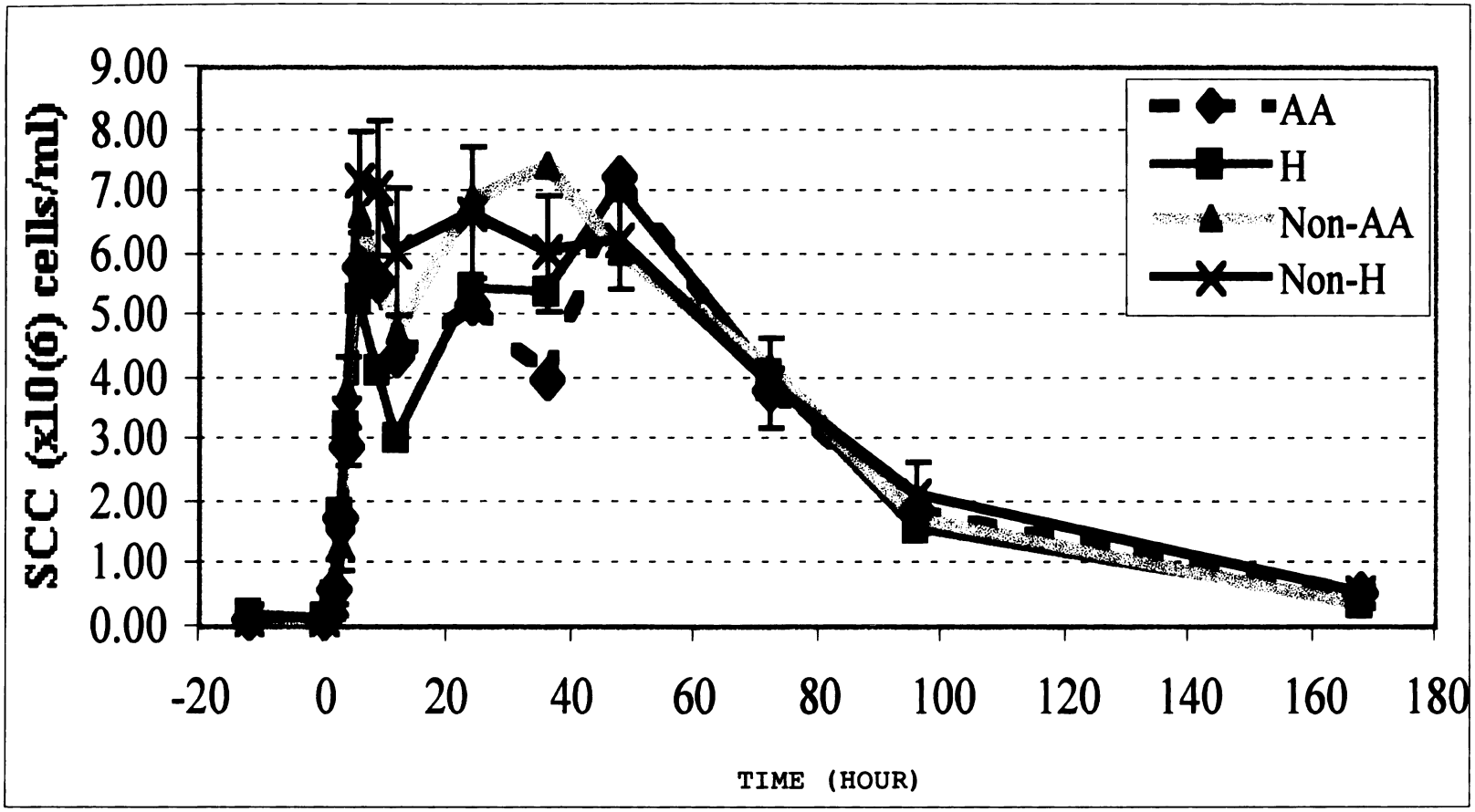

Figure 3 Mean Somatic Cell Count (SCC) as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. Mean SCC in AA cows is significantly lower than in non-AA cows at 24-36 hour post-LPS challenge ($P = 0.0261$). Moreover, from 6 to 24 hours after challenge. Mean SCC in H cows is significantly lower than in non-H cows from 6 to 24 hour after LPS challenge ($P = 0.0164$).

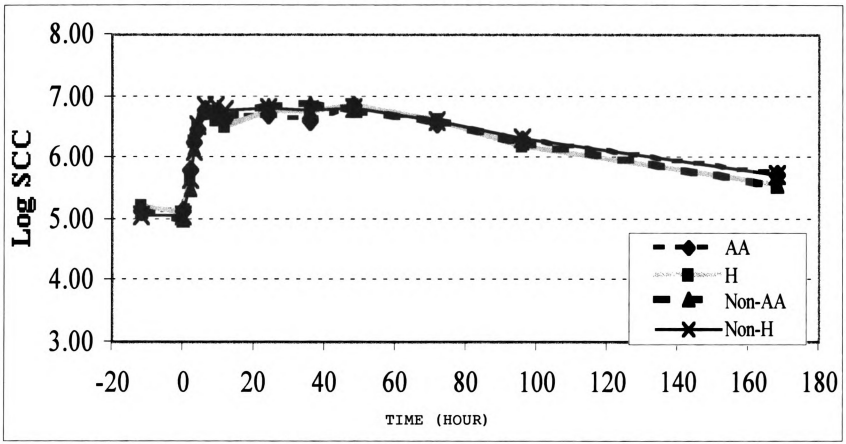

Figure 4 Logarithmical Somatic Cell Count (SCC) as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle.

III. Quarter Milk production

There was no significant difference between AA and non-AA cows, and H and non-H cows throughout the trial. Mean quarter milk production tended to be higher in H cows as compared to non-H cows at 12 hour post LPS challenge ($P = 0.0875$, Table 7). The data is plotted in Figures 5 and 6. Daily quarter milk production and daily change are shown in Figures 7 and 8.

Milk from non-challenged quarters remained normal both in appearance and bacteriologically negative throughout the period of study.

IV. Composite Milk production

The lowest amount of mean composite milk production (kg ± SEM) was at 12 hr post-LPS challenge for all treatment groups (AA = 7.28 ± 0.92, non-AA = 6.94 ± 0.81, H = 6.36 ± 0.86, and non-H = 6.70 ± 0.89) (Table 11). The mean milk production following AA treatments was significantly higher from 48 to 96 hr post-LPS challenge as compared to the milk production in the non-AA treatments ($P < 0.02$, Table 11). Although mean composite milk production tended to be higher in the non-H treatments than in the H treatments especially at 24 hr post-LPS challenge (non-H = 8.98 ±1.07 kg, H =8.29 ± 0.83 kg). There was no significant difference at any milking time. The data is plotted in Figures 9-12.

V. Milk IgG_1

Mean IgG_1 concentration (mg/ml) in AA cows tended to be higher than in non-AA cows at 6 and 24 hours after LPS challenge ($P < 0.10$, Table 15). Mean milk IgG_1 concentrations following H treatment peaked at 12 hr (1.82 ± 0.22 mg/ml), and was lower

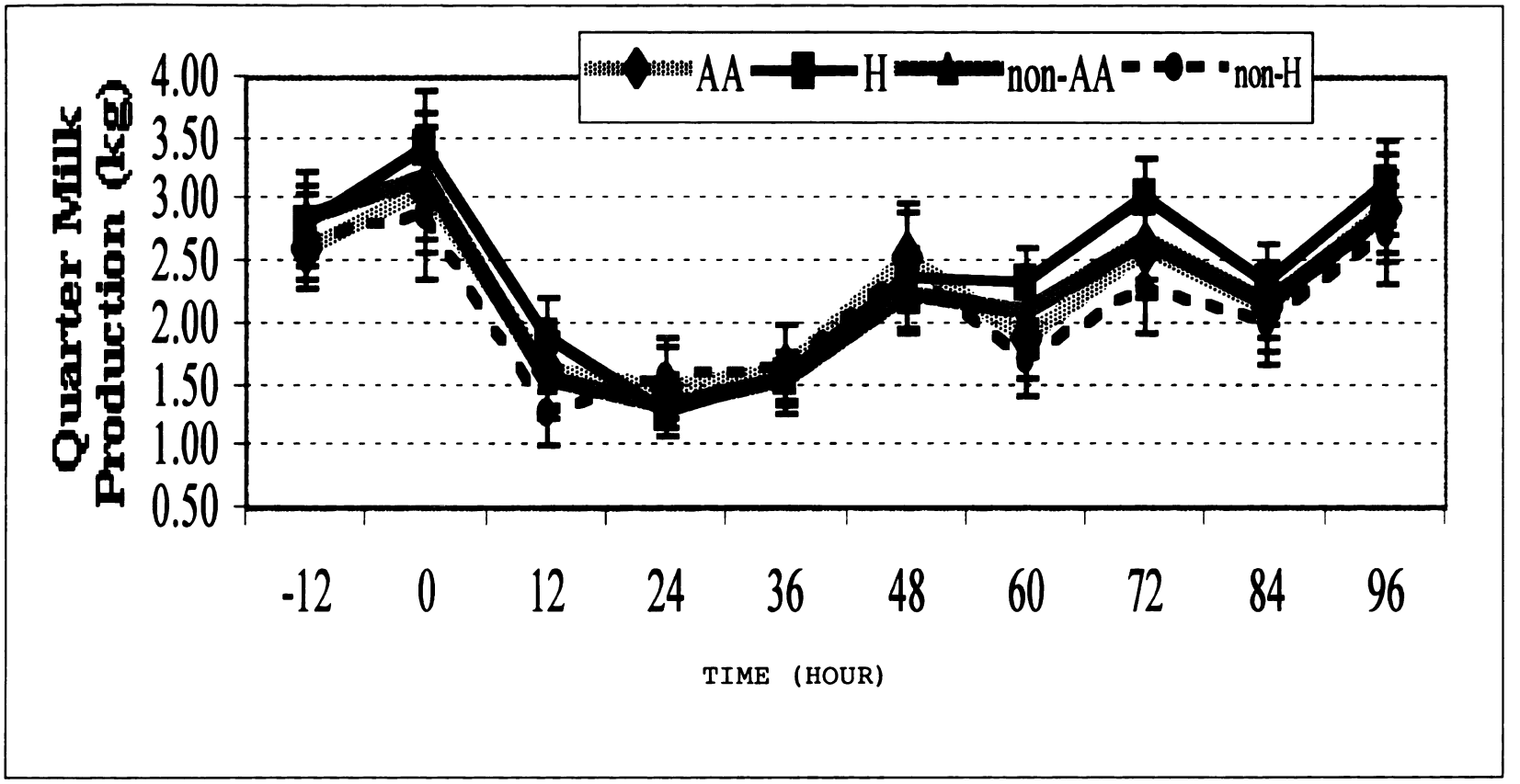

Figure 5 Mean Quarter Milk Production as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. There are no significant differences between AA and non-AA cows throughout the trial. Mean quarter milk production in H cows tended to be higher than in non-H cows at 12 hours post LPS challenge ($P < 0.090$).

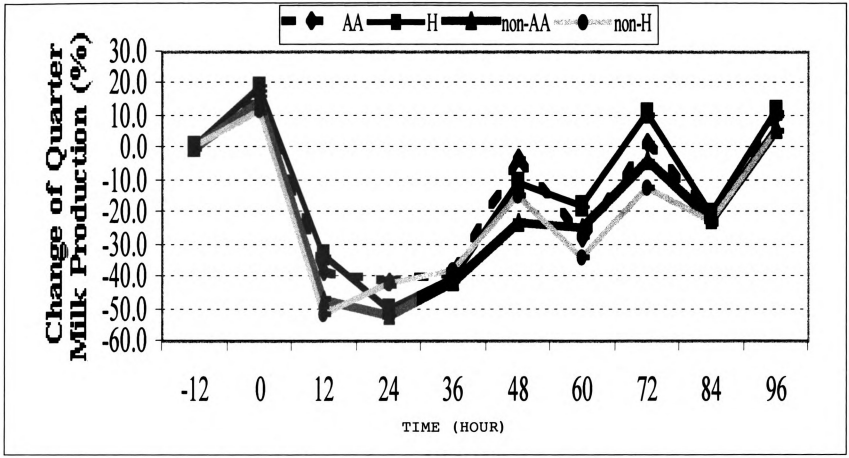

Figure 6 Change of Mean Quarter Milk Production as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. There are no significant differences between AA and non-AA cows throughout the trial. Mean quarter milk production in H cows tended to be higher than in non-H cows at 12 hours post LPS challenge ($P < 0.090$).

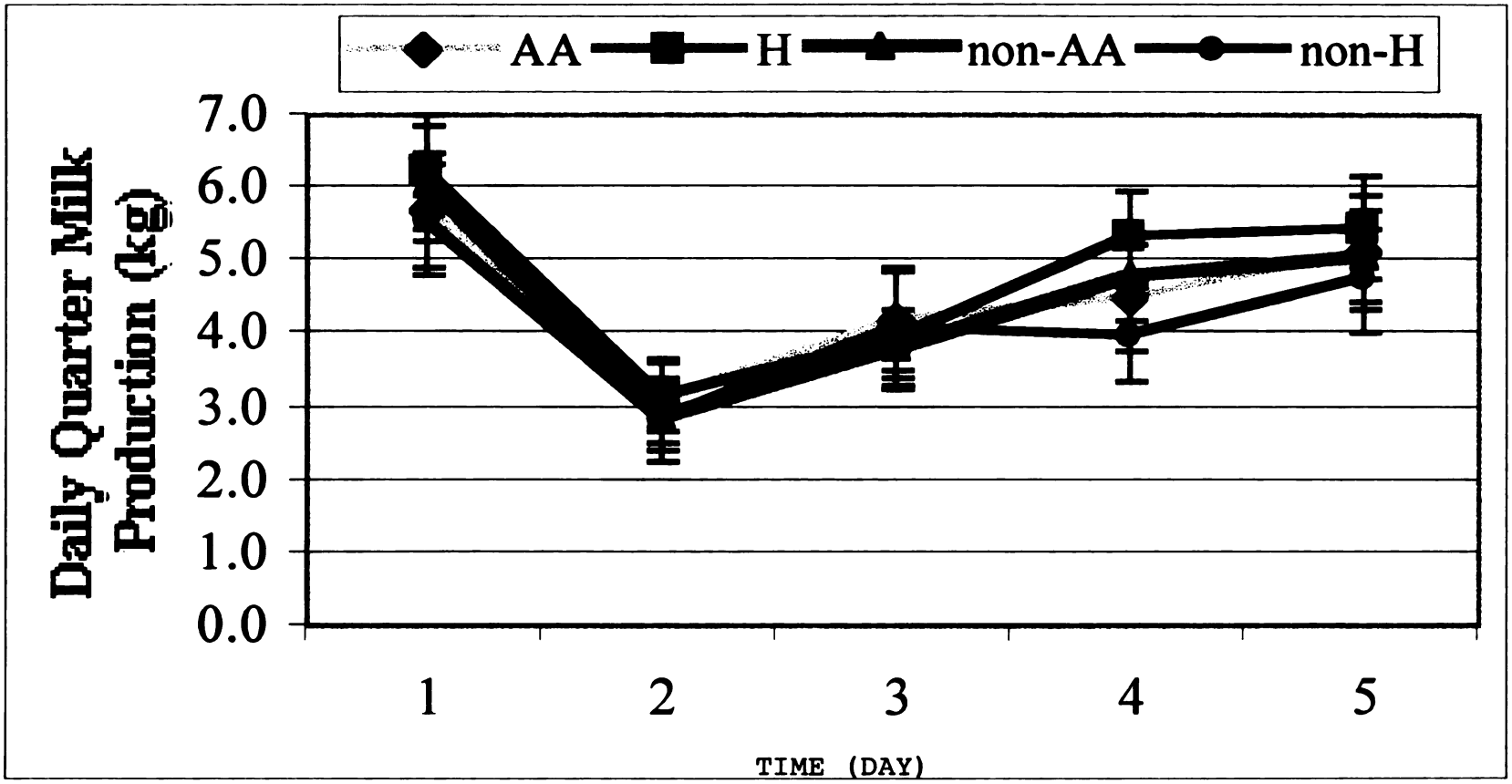

Figure 7 Daily Mean Quarter Milk Production as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. There are no significant differences between AA and non-AA cows throughout the trial.

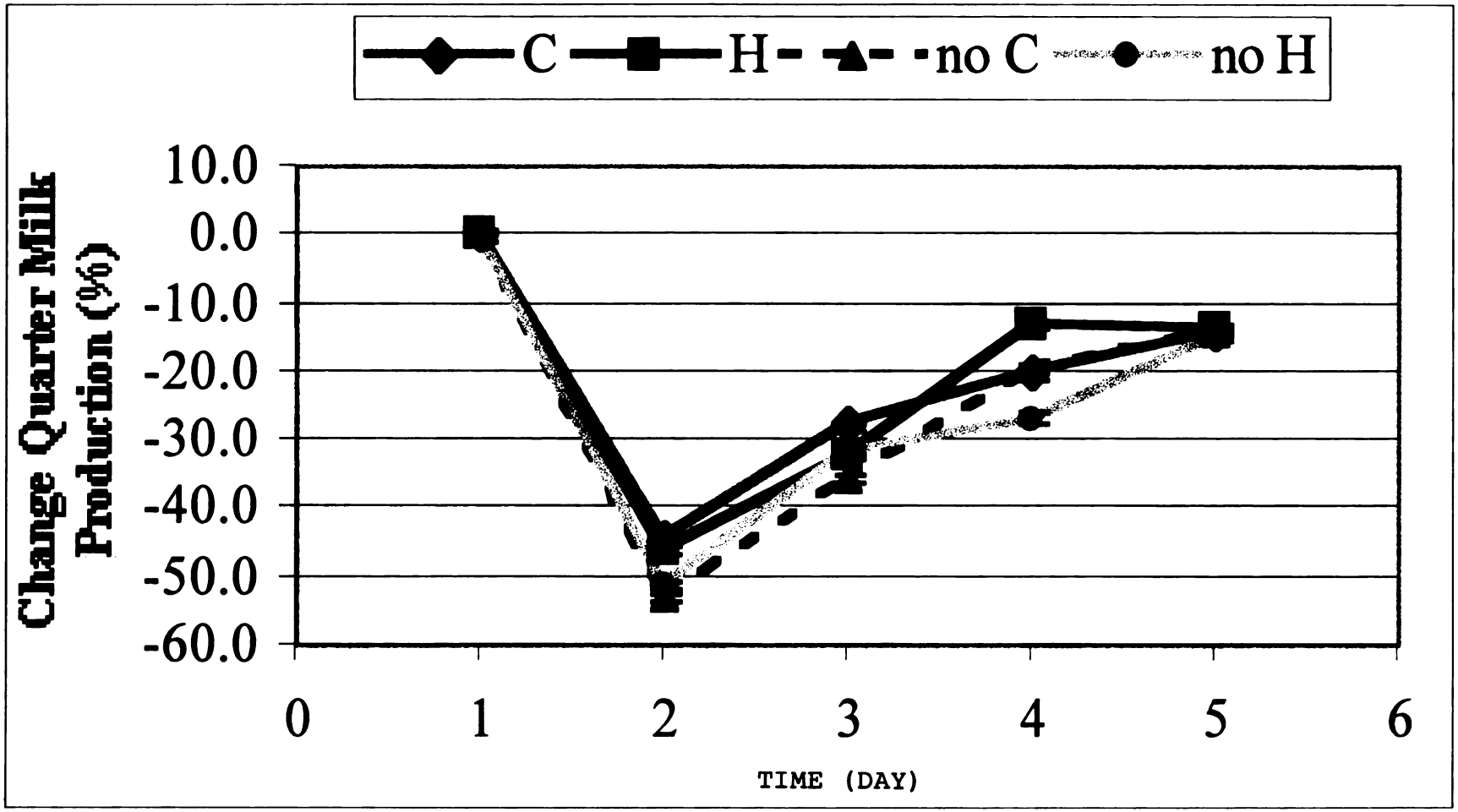

Figure 8 Change of Daily Mean Quarter Milk Production as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. There are no significant differences between AA and non-AA cows throughout the trial.

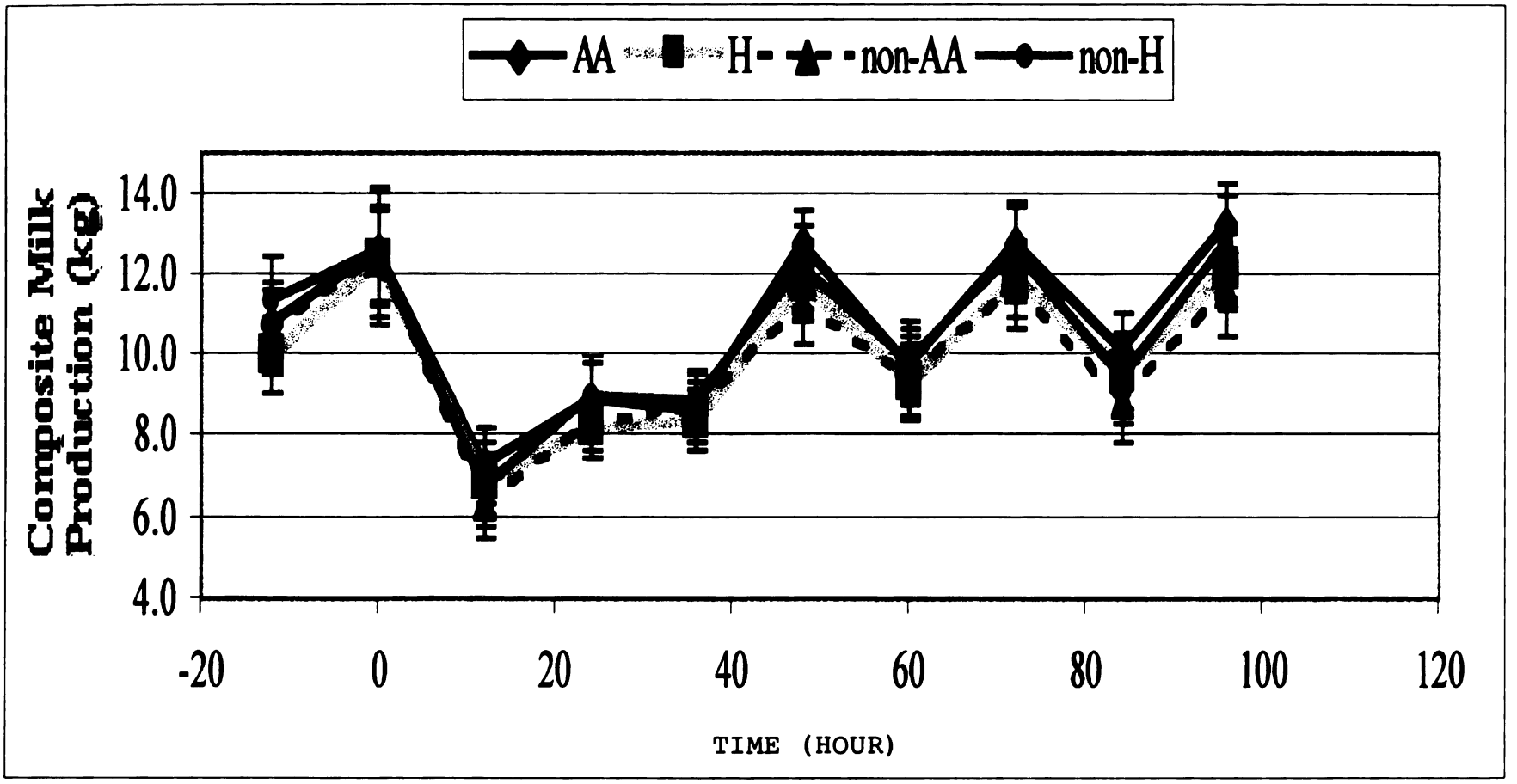

Figure 9 Mean Composite Milk Production as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. Mean composite milk production in AA cows is significantly higher than in non-AA cows from 48 to 96 hours post LPS challenge ($P < 0.02$). Whereas there are no significant differences between H and non-H cows throughout the trail.

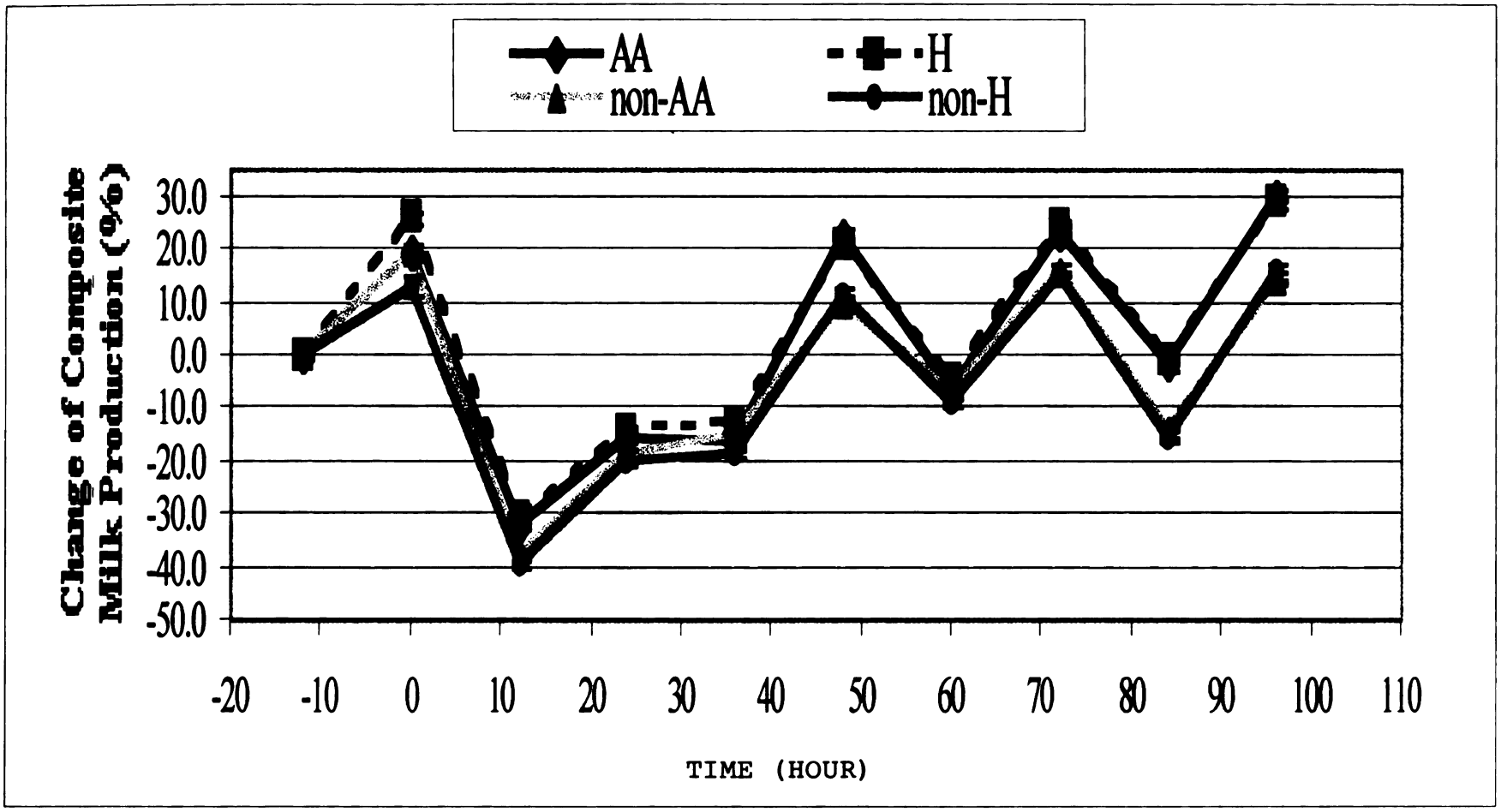

Figure 10 Change of Mean Composite Milk Production as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. Mean composite milk production in AA cows is significantly higher than in non-AA cows from 48 to 96 hours post LPS challenge ($P < 0.02$). Whereas there are no significant differences between H and non-H cows throughout the trail.

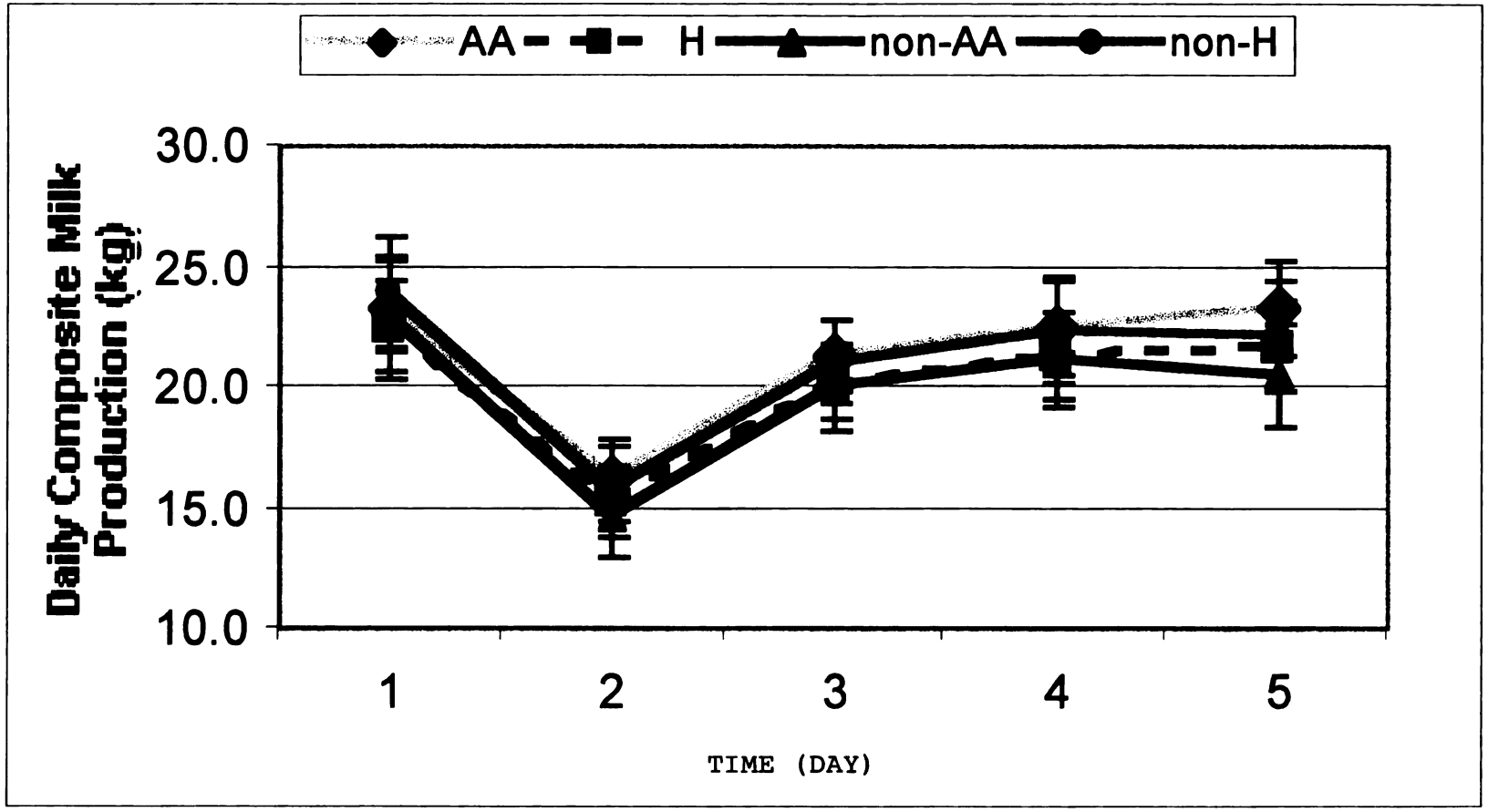

Figure 11 Daily Mean Composite Milk Production as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. There are no significant differences between H and non-H cows throughout the trail.

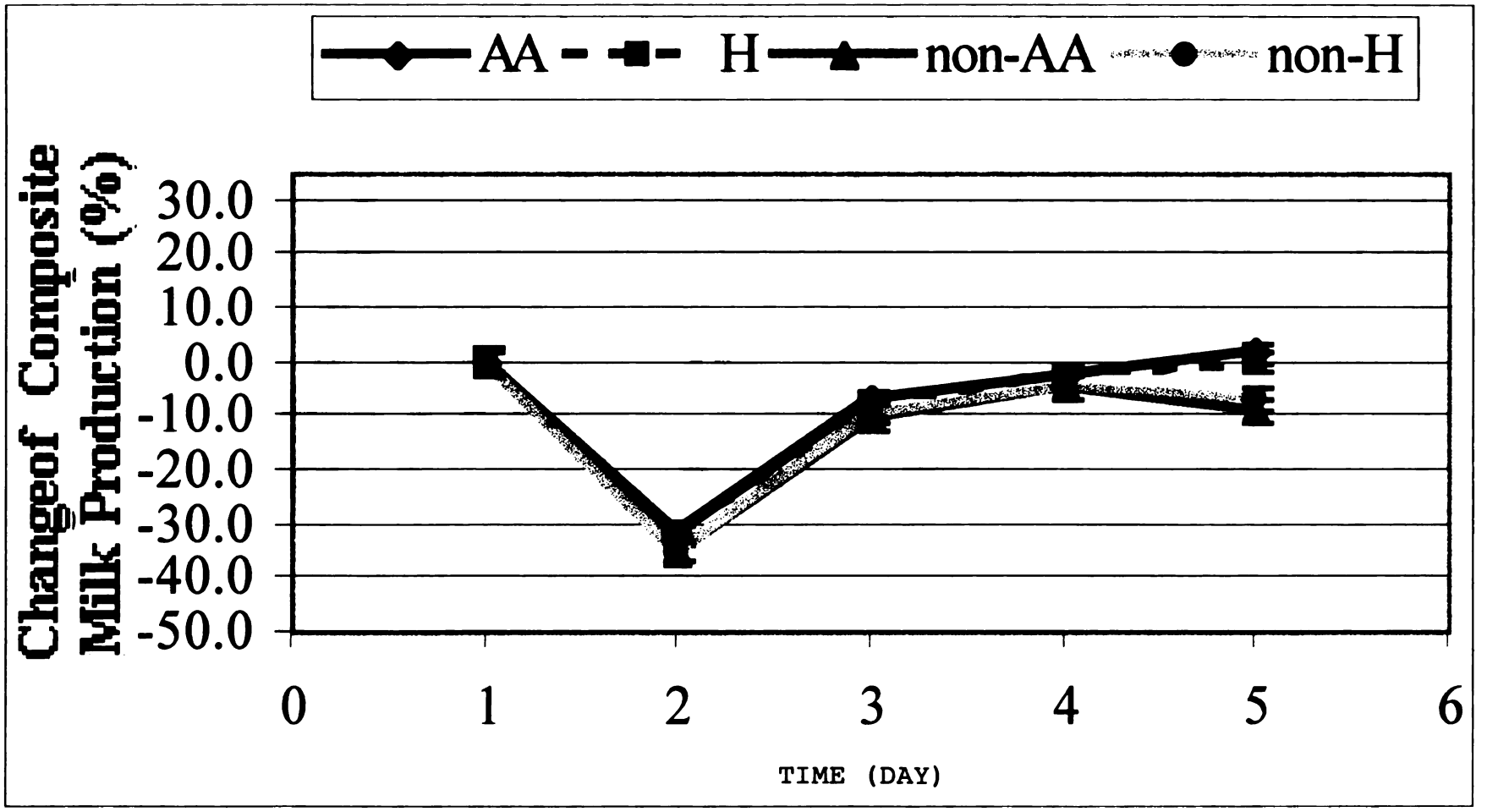

Figure 12 Change of Daily Mean Composite Milk Production as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. There are no significant differences between H and non-H cows throughout the trail.

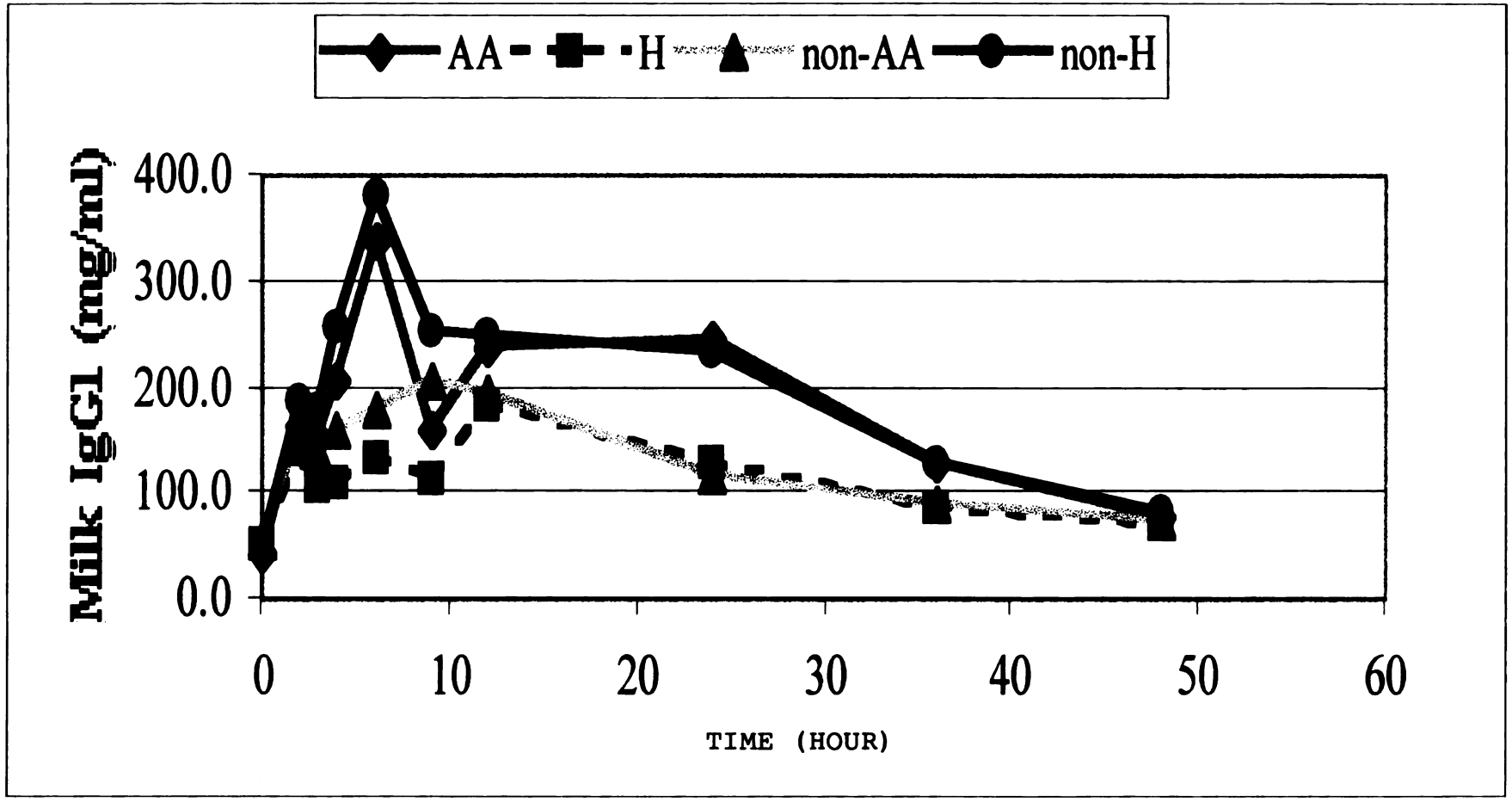

Figure 13 Mean Milk IgG_1 as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. Mean milk IgG_1 in AA cows tended to be higher than in non-AA cows at 6 and 24 hours after LPS challenge ($P < 0.10$). Mean milk IgG_1 in H cows also tended to be lower than in non-H cows from 3 to 9 hours post LPS challenge ($P = 0.0555$).

($P = 0.0555$, Table 15) than in non-H treatments from 3 hr to 9 hr post-LPS challenge. The data is plotted in Figure 13.

VI. Antioxidant Activities (AOA)

Mean antioxidant activities (% Inhibition) in H cows peaked (62.95 ± 1.92) at 6 hr post-LPS challenge, and were lower than in non-H cows (69.21 ± 2.26, 64.69 ± 2.02, 65.07 ± 1.95) from 6 to 12 hr post-LPS challenge ($P < 0.04$, Table 17). There was no significant difference between AA and non-AA cows at any time after LPS challenge. The data is plotted in Figure 14.

VII. Plasma Ascorbic Acid

Mean ascorbic acid concentration (μM/L) rapidly increased after the first infusion, peaked after the second infusion at 6 hr post-LPS challenge, and rapidly dropped afterward (Table & Figure 15).

VIII. Heart Rate (HR)

Mean heart rate was not affected by AA or L-histidine throughout the experimental period (Table 20). Mean rectal temperature in period 1 was significantly lower than in period 2 and 3 from 0 to 4 hr post LPS challenge ($P < 0.04$, Figure 2). Mean HR in period 1 was also significantly higher than in other periods from 0 to 2 hr post LPS challenge ($P < 0.05$). The data is plotted in Figure 16.

IX. Respiratory Rate (RR)

Mean RR did not differ between AA and non-AA cows, and H and non-H throughout the trial (Table 22). Mean HR in period 1 was also significantly higher than in period 2 and 3 after 4 hr post LPS challenge ($P < 0.02$). Mean HR in period 1 was

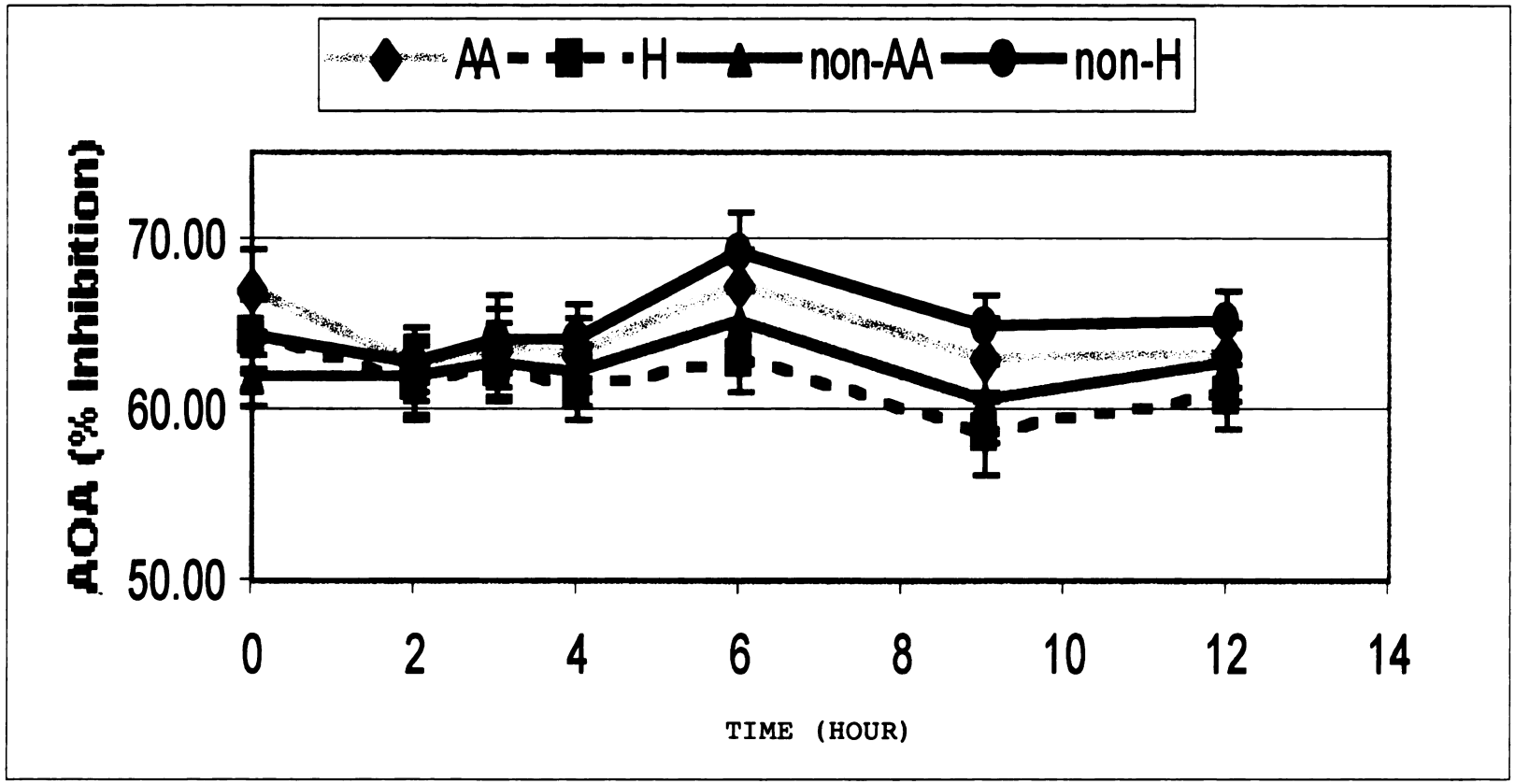

Figure 14 Mean Antioxidant Activities (AOA, % inhibition) as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. Mean AOA in H cows is significantly lower than in non-H cows from 6 to 12 hours post-LPS challenge ($P < 0.04$). Whereas there were no significant differences of mean AOA between AA and non-AA cows at any time.

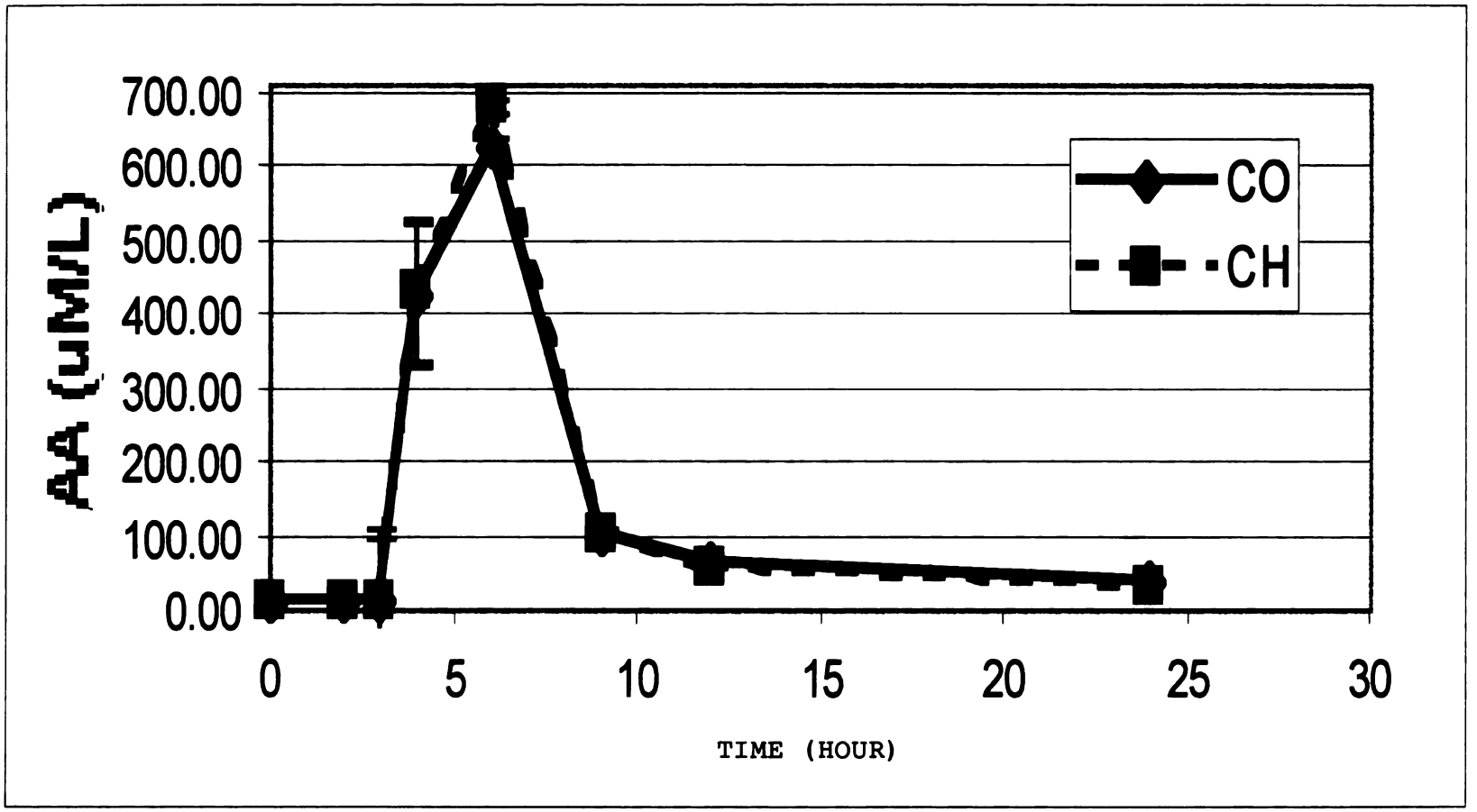

Figure 15 Mean Plasma Ascorbic Acid concentration by Hours Following LPS Challenge in CO (n = 8): LPS + Ascorbic acid, and CH (n = 8): LPS + Ascorbic acid + L-histidine in dairy cattle. There is no significant difference between CO and CH after ascorbic acid infusion.

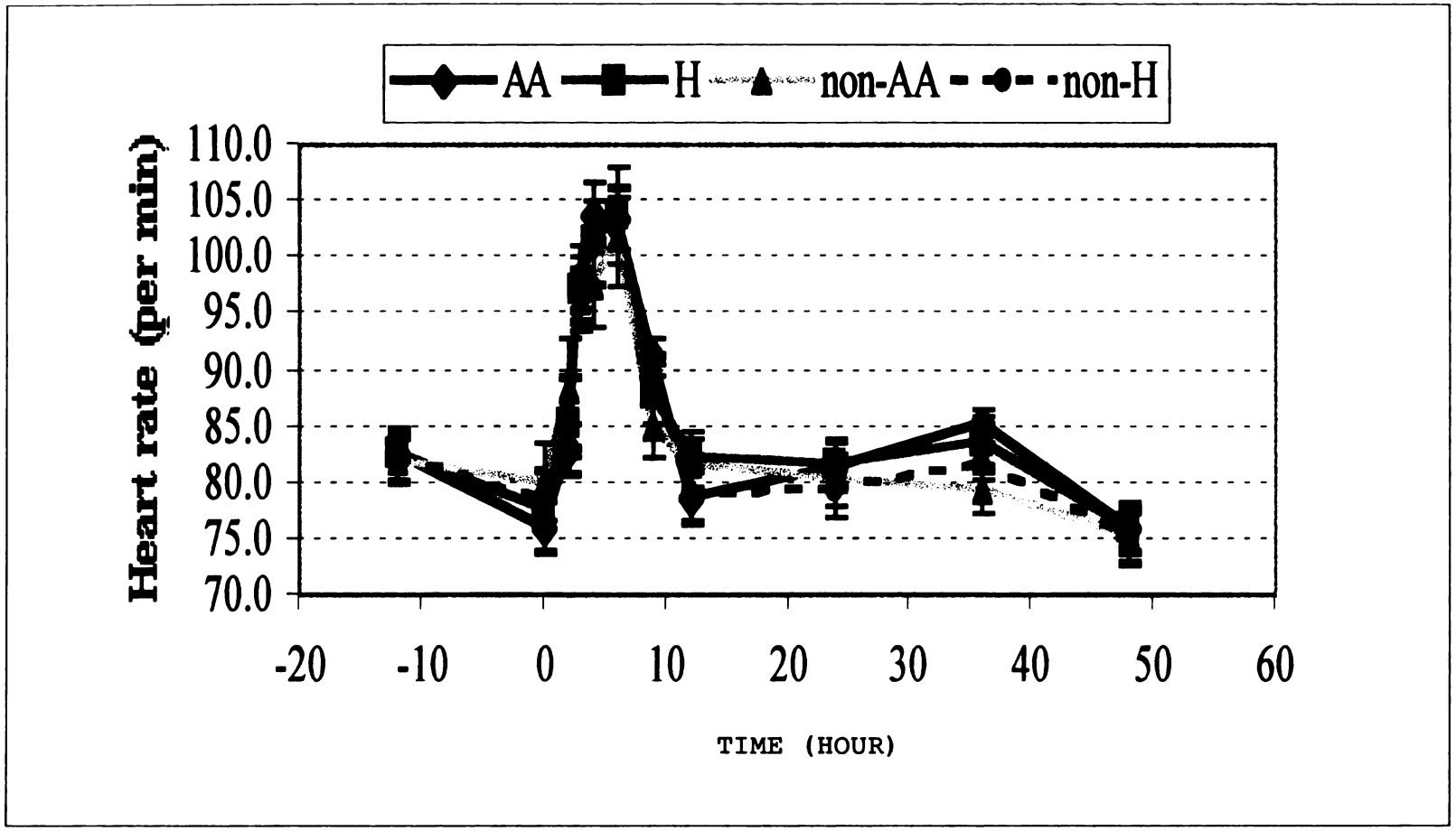

Figure 16 Mean Heart Rate as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. There were no significant differences between AA and non-AA treatments, as well as between H and non-H treatments.

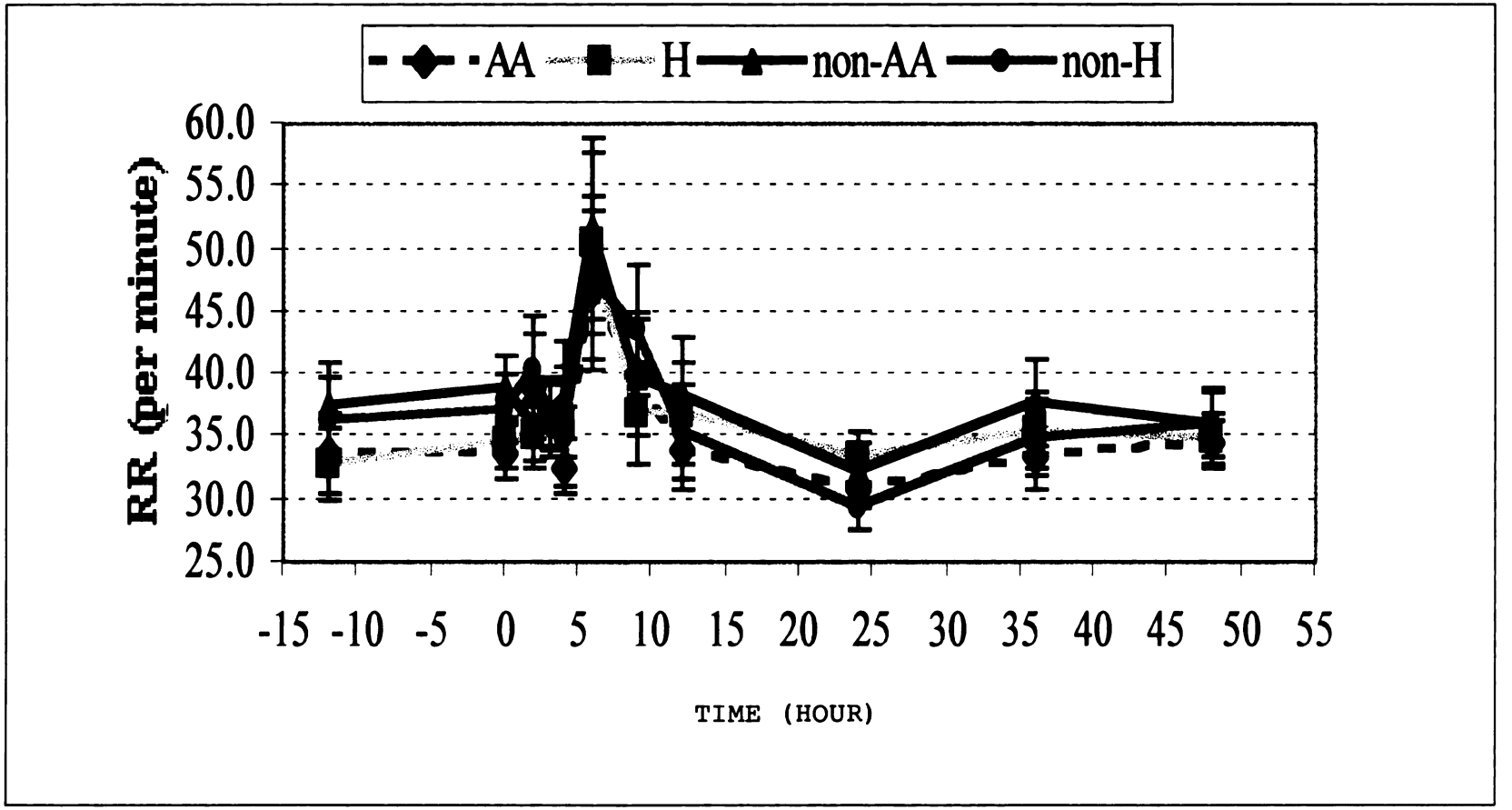

Figure 17 Mean Respiratory Rate (per minute) as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. No significant difference between AA and non-AA cows, also between H and non-H cows.

significantly lower than in period 2 and 3 after 4 hr post LPS challenge ($P < 0.02$). Mean HR in period 2 also differed from HR in period 4 after 4 hr post LPS challenge ($P < 0.03$). The data is plotted in Figure 17.

X. Ruminal Contraction Rate (RCR)

Mean RCR in AA cows tended to be higher ($P < 0.10$, Table 21) than in non-AA cows. There was no significant difference between H and non-H cows at any time. The data is plotted in Figure 18.

XI. Dry Matter Intake

Compared to the one day before LPS challenge (D-1), the mean percentage decreased DMI in all treatments markedly decreased in post challenge (Figure 20 & Table 28). There were no significant differences between AA and non-AA cows, as well as between H and non-H cows. The data is plotted in Figures 19-20.

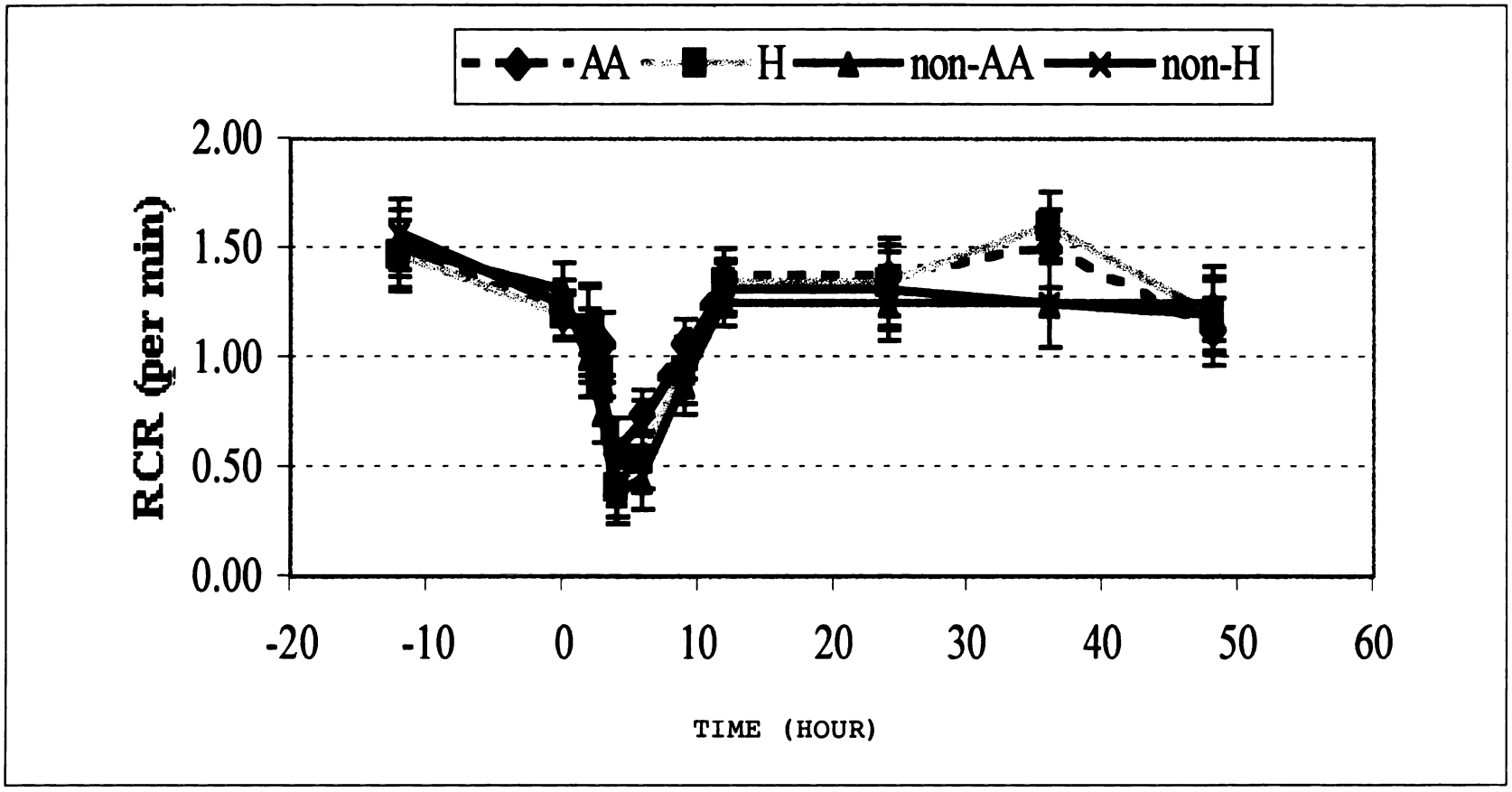

Figure 18 Mean Ruminal Contraction Rate (per minute) as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. Mean ruminal contraction rate in AA cows tended to be higher than in non-AA cows from 3 to 6 hours post-LPS challenge ($P < 0.10$). No significant difference between H and non-H cows at any time.

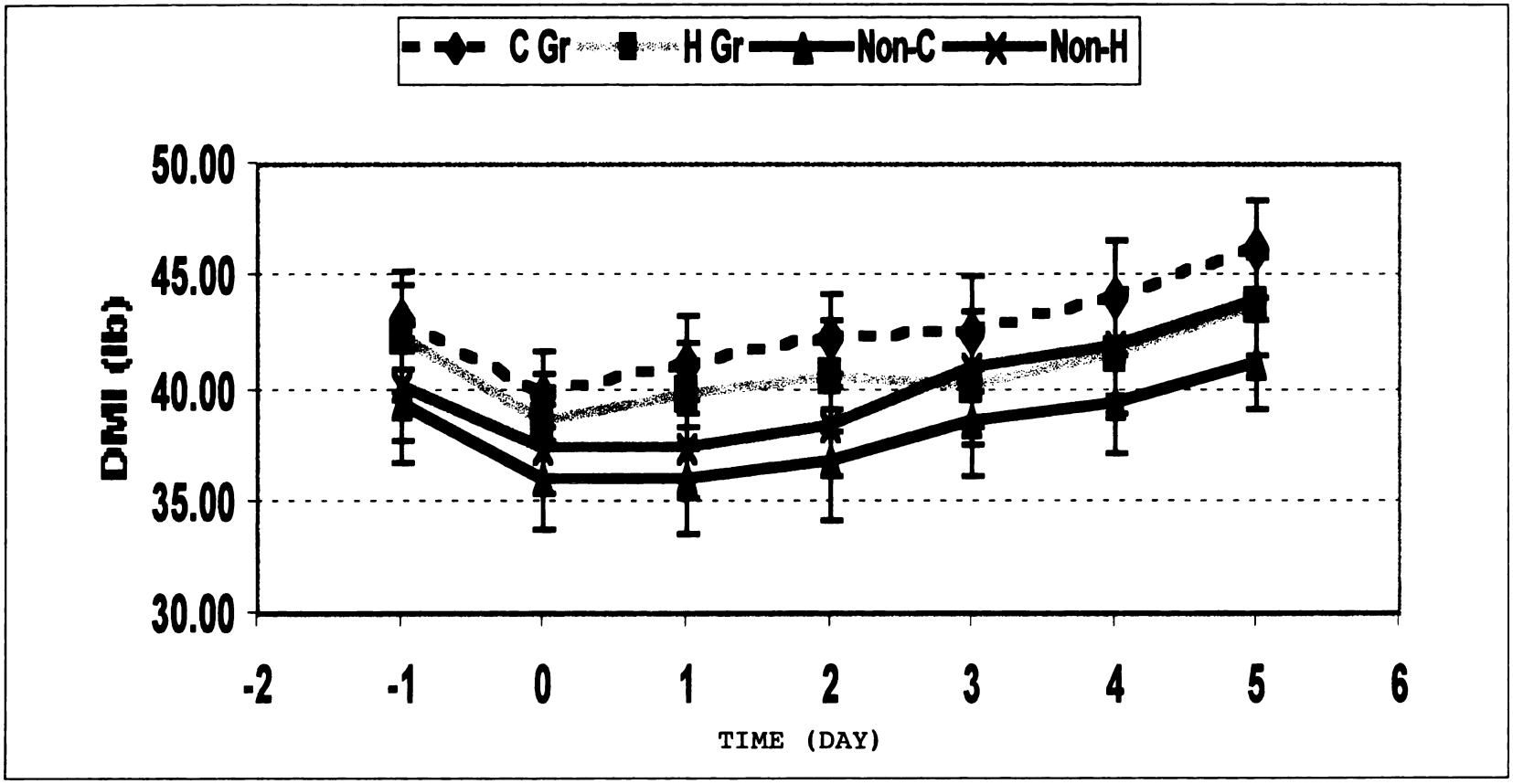

Figure 19 Mean Dry Matter Intake (lbs.) as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. Mean DMI in AA had been statistically significant higher ($P < 0.06$) than in No AA since the beginning, but there was no significant difference between H and non-H cows H.

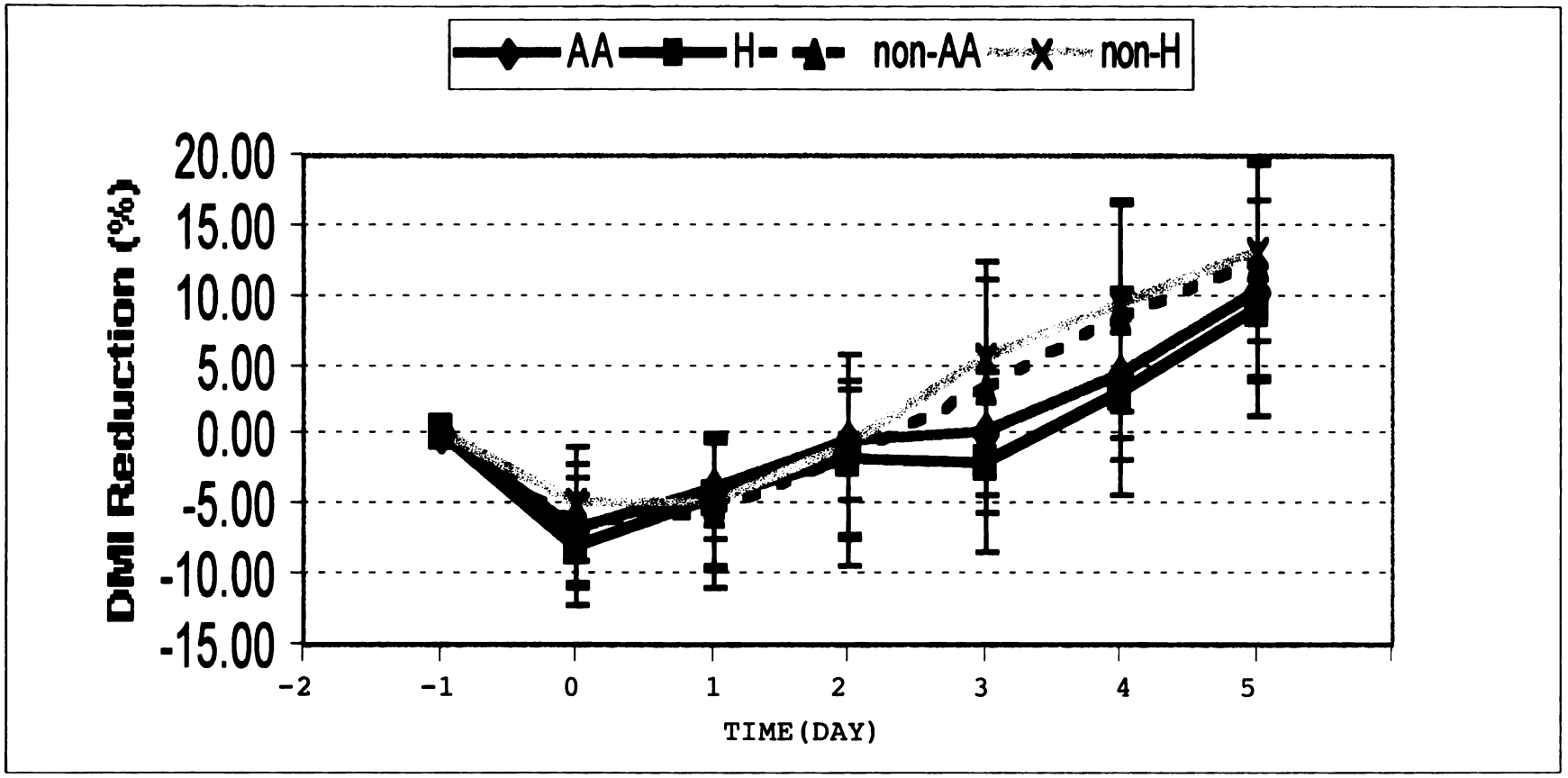

Figure 20 Mean Dry Matter Intake (% Reduction) as Treatment Groups; AA (n = 16): CO + CH, H (n = 16): OH + CH, No AA (n = 16): OO + OH, No H (n = 16): OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + L-histidine), and CH (LPS + Ascorbic acid + L-histidine) in dairy cattle. No significant difference between AA and No AA, also between H and non-H cows.

DISCUSSION

Endotoxin-induced mastitis is widely accepted as an excellent model of acute mammary inflammation (Shuster et al., 1991a, 1991c; Shuster & Harmon, 1991) because of the reversible effect of endotoxin on the mammary gland parenchyma (Schalm, 1977) and a self-limited response (Shuster & Harmon, 1993). Therefore, this model is clinically applicable for use in the determination of the effect of L-His and ascorbic acid therapy on acute mammary inflammation. In this study design, variables including rectal temperature, somatic cell count, milk production (quarter & composite), milk IgG_1, antioxidant activities (AOA), plasma ascorbic acid, heart rate, respiratory rate, ruminal contraction rate, and dry matter intake were used to determine the magnitude of acute mammary inflammation.

The Latin square cross-over design allowed for use of fewer experimental subjects. However, there are two important drawbacks with this design; carryover effects and washout periods (Fleiss, 1986; Zar, 1996). The effect of the treatment given in one period might carry over into the next period and thus obscure the effect of subsequent treatments if a sufficient time was not allowed (a washout period) between the administration of different treatments (Fleiss, 1986; Zar, 1996). However, this potential problems was not likely in our study because both ascorbic acid and L-His had a short half-life (Peterson et al., 1998; Sitton et al., 1988); particularly for ascorbic acid since the serum concentration peaked and decreased to the pre-treatment levels within hours after infusion.

In this study, rectal temperature rose after IMM administration of endotoxin aslso

seen in previous studies (Jackson et al., 1990; Lohuis et al., 1988; Shuster et al., 1993). The mechanism of fever might be explained by the action of IL-1 on thermoregulatory centers within the hypothalamus by stimulating PGE_2 synthesis (Lohuis et al., 1988). PGE_2 itself removes inhibition of thermosensitive neurons and eventually results in a sharp increase in body temperature (Lohuis et al., 1988). The period effect on rectal temperature may have been influenced by environmental temperature in the barn. Unfortunately, we cannot provide data to support this point.

Previous studies demonstrated the tolerance to *E. coli* endotoxin induced by repetitive daily intravenous (Lohuis et al., 1988) or intramammary administration (Shuster & Harmon, 1991). Cows infused with 10 μg of *E. coli* endotoxin in the same two homolateral quarters twice daily for several days became partially refractory to subsequent infusions in terms of systemic, but not local, effects (Shuster & Harmon, 1991). This phenomenon was not seen in this study, which was conducted by endotoxin infusion at weekly intervals. Milk production completely recovered before the beginning of each consecutive treatment. It is possible that in the previous study that dose of 10-μg of intramammary endotoxin infusion was too a small dose to induce enough inflammatory mediators for a systemic response. Generally, it only causes a mild to moderate mastitic and systemic response (Shuster et al., 1993; Shuster & Harmon, 1991).

Based on the SCC data in this study, L-His and ascorbic acid seemed to reduce SCC between 6 and 48 hr post-LPS challenge (Figure 3). However, the accuracy of SCC was questionable. Because of the severe abnormality and the presence of many flakes and clots in the milk during that time, SCCs were subjected to a great deal of variability. This

event commonly occurs during acute mastitis because fibrinogen that has diffused from blood plasma into milk is converted into fibrin strands that enmesh leukocytes, epithelial cells and other debris, and eventually forms the flakes and clots (Schalm, 1977).

Pathological changes in milk result from an increase capillary permeability with an outflow of plasma proteins such as bovine serum albumin (BSA) and IgG (Kitchen, 1981; Schalm, 1977). In this study, we used IgG_1 as an indicator of a mammary inflammation rather than BSA or both BSA and IgG_1. Additionally, we believe milk concentration of IgG_1 provides a more accurate measure of inflammation than SCC in an acute mastitis model.

Although evidence supports L-His as an antioxidant *in vitro* and in disease models in laboratory animals (Kawamoto et al., 1997; Nagy & Floyd, 1984; Peterson et al., 1998). L-His decreased plasma AOA (Figure 11 and 12) in this study. *In vitro,* studies demonstrated that L-His triggered cellular (particularly DNA) damage and cytotoxicity in mammalian cells mediated by H_2O_2 (Cantoni et al., 1992; Guidarelli et al., 1995; Tachon et al., 1994). In an environment concomitant with H_2O_2 presence, parenteral L-His administration may not function as an antioxidant, but rather as an oxidative catalyst.

In contrast, parenteral ascorbic acid administration tended to increase plasma AOA, which simultaneously corresponded to the plasma ascorbic acid concentration particularly at 6 hr post LPS challenge (Figure & Table 11). Ascorbic acid might provide a good benefit on acute mammary inflammation with high AOA to quench free radicals, and singlet oxygen (Bodannes et al., 1979; Niki, 1991b) by providing hydrogen atoms to pair up with unpaired electrons on free radicals (Jacob, 1995).

In this study, an increase in AOA following LPS challenge might be explained through the role other antioxidant mechanisms in cellular oxidative metabolism resulting from phagocytic activation and acute inflammation. For example, sequestration of transition metals, particularly Fe, may be a mechanism to reduce generation of oxygen radicals (Erskine, 1993; Halliwell, 1987). In *E. coli*-induced mastitis cows, mean serum concentrations of Fe decreased 28-35% (Erskine & Bartlett, 1993; Lohuis et al., 1988).

As in previous studies, hypogalactia mediated by systemic and local effects of acute mammary inflammation completely recovered after inflammation subsided (Shuster et al., 1991, 1991a, 1991c; Shuster & Harmon, 1991). Significantly higher milk production after 48-hr post LPS challenge following ascorbic acid treatment was consistent with a potential beneficial effect of AA treatment.

Experimental endotoxin intramammary infusion or field cases of *E. coli* mastitis have been reported to not decrease rumen motility (Lohuis et al., 1988; Verheijden et al., 1983). However, we determined decreased rumen motility in our study, which is consistent with decreased rumen motility, amplitude and frequency in other studies of clinical mastitis caused by gram-negative bacteria (Lohuis et al., 1990; Morin et al., 1998).

In conclusion, treatment of cows with ascorbic acid following endotoxin-induced mastitis increased recovery of milk production, and helped to maintain DMI. However, the outcomes are prone to much variation. This study might have been conducted on too few cattle to determine conclusive results. Additionally, endotoxin challenge may not cause a sufficiently severe mastitis to attain differences in outcome variables between treatments as compared to an *E. coli* challenge. Further research is necessary to fully

understand and elucidate the role of L-His and ascorbic acid on acute mammary inflammation in dairy cattle.

APPENDICES

APPENDIX A

Table 1 Mean Rectal Temperature by Hours Following LPS Challenge in Four Different Treatments; OO: LPS alone, CO: LPS + Ascorbic acid, OH: LPS + Histidine, and CH: LPS + Ascorbic acid + Histidine in dairy cattle.

Mean Rectal Temperature (°F)								
Time	**OO**		**CO**		**OH**		**CH**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**101.33**	0.34	**101.63**	0.18	**101.70**	0.26	**101.28**	0.17
0	**101.70**	0.56	**101.73***	0.62	**101.54**	0.29	**100.86**	0.74
2	**102.79**	0.49	**103.30***	0.80	**102.58**	0.59	**102.49**	0.53
3	**104.26**	0.52	**103.95**	0.84	**104.00**	0.61	**104.01**	0.53
4	**104.95**	0.54	**104.40**	0.67	**104.99**	0.57	**104.63**	0.62
6	**104.90**	0.47	**105.09**	0.40	**105.41**	0.49	**104.64**	0.54
9	**101.53**	0.58	**102.43**	0.44	**102.28**	0.52	**102.10**	0.55
12	**100.89**	0.46	**100.94**	0.30	**101.31**	0.23	**100.88**	0.22
24	**100.45*****	0.34	**99.78**	0.25	**100.00**	0.19	**99.64**	0.32
36	**100.83**	0.27	**101.01**	0.27	**101.81****	0.45	**100.98**	0.10
48	**101.29*****	0.41	**101.29***	0.24	**100.98**	0.32	**100.61**	0.21

The data are expressed as means (±SEM) and based on 8 observations/treatment group.

* Mean rectal temperature in CO tends to be higher than in CH ($P < 0.10$).

** Mean rectal temperature in OH is significantly higher than in all other treatments ($P < 0.040$).

*** Mean rectal temperature in OO is significantly higher than in CO and CH ($P < 0.050$).

Table 2 Mean Rectal Temperature as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Mean Rectal Temperature (°F)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**101.42**	0.12	**101.51**	0.21	**101.49**	0.16	**101.44**	0.19
0	**101.35**	0.49	**101.62**	0.31	**101.20**	0.40	**101.77**	0.41
2	**103.00**	0.46	**102.68**	0.37	**102.53**	0.38	**103.15**	0.44
3	**104.12**	0.48	**104.13**	0.39	**104.01**	0.39	**104.24**	0.47
4	**104.66**	0.46	**104.97**	0.38	**104.81**	0.41	**104.82**	0.44
6	**104.89**	0.34	**105.16**	0.33	**105.03**	0.36	**105.02**	0.31
9	**102.34**	0.35	**101.90**	0.39	**102.19**	0.37	**102.05**	0.38
12	**100.88**	0.18	**101.10**	0.25	**101.09**	0.16	**100.88**	0.26
24	**99.67***	0.19	**100.23**	0.19	**99.82**	0.19	**100.08**	0.22
36	**100.98***	0.14	**101.32**	0.28	**101.39**	0.25	**100.90**	0.19
48	**100.89***	0.16	**101.13**	0.25	**100.79**	0.19	**101.23**	0.23

The data are expressed as means (±SEM) and based on 16 observations per treatment groups.
* Mean rectal temperature AA cows is significantly lower than in non-AA cows (P = 0.0393).
No significant difference between H and non-H treatments at any time.

Table 3 Change of Mean Rectal Temperature as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments: OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Change of Mean Rectal Temperature (°F)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**0.00**	0.00	**0.00**	0.00	**0.00**	0.00	**0.00**	0.00
0	**-0.07**	0.48	**0.11**	0.31	**-0.29**	0.36	**0.32**	0.44
2	**1.58**	0.48	**1.17**	0.43	**1.04**	0.39	**1.71**	0.50
3	**2.70**	0.47	**2.62**	0.44	**2.52**	0.39	**2.80**	0.52
4	**3.24**	0.44	**3.46**	0.42	**3.32**	0.39	**3.38**	0.47
6	**3.47**	0.34	**3.64**	0.33	**3.54**	0.35	**3.58**	0.31
9	**0.92**	0.38	**0.39**	0.40	**0.70**	0.42	**0.61**	0.37
12	**-0.54**	0.21	**-0.41**	0.24	**-0.39**	0.19	**-0.56**	0.26
24	**-1.75***	0.24	**-1.29**	0.26	**-1.67**	0.20	**-1.37**	0.30
36	**-0.44***	0.17	**-0.19**	0.27	**-0.09**	0.22	**-0.54**	0.22
48	**-0.53***	0.17	**-0.38**	0.23	**-0.69**	0.19	**-0.22**	0.20

The data are expressed as means (±SEM) and based on 16 observations per treatment groups.
* Change of mean rectal temperature in AA cows is significantly lower than in non-AA cows (P = 0.0393). No significant difference between H and non-H cows at any time.

Table 4 Mean Somatic Cell Count (SCC) by Hours Following LPS Challenge in Four Different Treatments: OO: LPS alone, CO: LPS + Ascorbic acid, OH: LPS + Histidine, and CH: LPS + Ascorbic acid + Histidine in dairy cattle.

Mean Somatic Cell Count ($\times 10^6$ cells/ml)								
Time	**OO**		**CO**		**OH**		**CH**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**0.08**	0.02	**0.15**	0.07	**0.19**	0.06	**0.13**	0.03
0	**0.08**	0.03	**0.15**	0.08	**0.12**	0.03	**0.12**	0.04
2	**0.40**	0.12	**0.49**	0.17	**0.20**	0.11	**0.74**	0.34
3	**1.62**	0.70	**1.02**	0.40	**1.10**	0.44	**2.47**	1.13
4	**4.17**	1.41	**2.76**	1.10	**3.39**	1.26	**3.06**	1.23
6	**7.85**	1.01	**6.48**	1.29	**5.42****	1.51	**5.13*****	1.54
9	**7.05**	1.59	**7.12**	1.60	**4.24****	1.62	**3.95*****	1.54
12	**6.65**	1.58	**5.47***	1.42	**2.91****	1.34	**3.17*****	1.58
24	**8.28**	1.24	**5.06***	1.63	**5.54****	1.57	**5.35*****	1.48
36	**8.13**	0.89	**3.89***	1.37	**6.69****	1.15	**4.11*****	1.38
48	**5.67**	1.22	**6.83**	1.03	**6.44**	1.12	**7.67**	0.92
72	**4.21**	1.11	**3.63**	0.99	**4.10**	0.97	**3.98**	1.11
96	**1.79**	0.52	**2.37**	1.11	**1.68**	0.18	**1.43**	0.31
1 week	**0.39**	0.11	**0.68**	0.27	**0.33**	0.07	**0.38**	0.14

The data are expressed as means (±SEM) and based on 8 observations/treatment group.

* Mean SCC in OO is significantly higher than in CO ($P = 0.0222$).

** Mean SCC in OO is significantly higher than in OH ($P = 0.0255$).

*** Mean SCC in OO is significantly higher than in CH ($P = 0.0058$).

Table 5 Mean Somatic Cell Count (SCC) as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Mean Somatic Cell Count ($\times 10^6$ cells/ml)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**0.14**	0.04	**0.13**	0.03	**0.16**	0.03	**0.12**	0.04
0	**0.13**	0.04	**0.10**	0.02	**0.12**	0.02	**0.11**	0.04
2	**0.61**	0.19	**0.30**	0.08	**0.47**	0.19	**0.45**	0.10
3	**1.75**	0.61	**1.36**	0.41	**1.79**	0.61	**1.32**	0.04
4	**2.91**	0.80	**3.78**	0.92	**3.22**	0.85	**3.47**	0.88
6	**5.81**	0.98	**6.63**	0.93	**5.27****	1.04	**7.17**	0.81
9	**5.54**	1.15	**5.65**	1.16	**4.09****	1.08	**7.09**	1.09
12	**4.32**	1.07	**4.78**	1.11	**3.04****	1.00	**6.06**	1.04
24	**5.20***	1.06	**6.91**	1.03	**5.45****	1.04	**6.67**	1.07
36	**4.00***	0.94	**7.41**	0.73	**5.40**	0.93	**6.01**	0.96
48	**7.25**	0.68	**6.05**	0.81	**7.06**	0.72	**6.25**	0.79
72	**3.80**	0.72	**4.16**	0.71	**4.04**	0.71	**3.92**	0.72
96	**1.90**	0.57	**1.74**	0.26	**1.55**	0.18	**2.08**	0.60
1 wk	**0.53**	0.15	**0.36**	0.06	**0.35**	0.07	**0.53**	0.15

The data are expressed as means (±SEM) and based on 16 observations/treatment group.

* Mean SCC in AA cows is significantly lower than in non-AA cows ($P = 0.0261$).

** Mean SCC in H cows is significantly lower than in non-H cows ($P = 0.0164$).

Table 6 Mean Quarter Milk Production by Hours Following LPS Challenge in Four Different Treatments; OO: LPS alone, CO: LPS + Ascorbic acid, OH: LPS + Histidine, and CH: LPS + Ascorbic acid + Histidine in dairy cattle.

Mean Quarter Milk Production (kg)								
Time	**OO**		**CO**		**OH**		**CH**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**2.79**	0.54	**2.60**	0.48	**2.97**	0.46	**2.62**	0.49
0	**2.52**	0.70	**3.22**	0.75	**3.88**	0.67	**2.96**	0.71
12	**0.95**	0.25	**1.61**	0.47	**2.16**	0.51	**1.62**	0.39
24	**1.20**	0.28	**1.91**	0.58	**1.47**	0.28	**1.05**	0.23
36	**1.32**	0.27	**1.94**	0.66	**1.82**	0.31	**1.29**	0.33
48	**2.06**	0.62	**2.79**	0.77	**2.41**	0.29	**2.31**	0.43
60	**1.75**	0.42	**1.64**	0.46	**2.48**	0.37	**2.14**	0.48
72	**2.22**	0.50	**2.36**	0.56	**3.14**	0.38	**2.88**	0.62
84	**1.90**	0.48	**2.11**	0.47	**2.48**	0.36	**2.19**	0.57
96	**2.56**	0.53	**2.90**	0.50	**3.23**	0.39	**3.01**	0.68

The data are expressed as means (± SEM) and based on 8 observations/treatment group.
No significant differences among treatments.

Table 7 Mean Quarter Milk Production as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Mean Quarter Milk Production (kg)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**2.61**	0.33	**2.88**	0.34	**2.79**	0.33	**2.69**	0.35
0	**3.09**	0.50	**3.20**	0.50	**3.42**	0.49	**2.87**	0.51
12	**1.62**	0.30	**1.55**	0.31	**1.89***	0.32	**1.28**	0.27
24	**1.48**	0.32	**1.33**	0.19	**1.26**	0.18	**1.55**	0.33
36	**1.62**	0.37	**1.57**	0.21	**1.56**	0.23	**1.63**	0.35
48	**2.55**	0.43	**2.24**	0.33	**2.36**	0.25	**2.43**	0.49
60	**1.89**	0.33	**2.12**	0.29	**2.31**	0.30	**1.70**	0.30
72	**2.62**	0.41	**2.68**	0.33	**3.01**	0.35	**2.29**	0.37
84	**2.15**	0.36	**2.19**	0.30	**2.33**	0.33	**2.01**	0.32
96	**2.95**	0.44	**2.89**	0.33	**3.12**	0.38	**2.73**	0.39

The data are expressed as means (±SEM) and based on 16 observations/treatment group.
There are no significant differences between AA and non-AA cows throughout the trial.
* Mean quarter milk production in H cows tended to be higher than in non-H cows ($P < 0.090$).

Table 8 Mean Daily Quarter Milk Production as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Mean Daily Quarter Milk Production (kg)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Day)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
1	**5.70**	0.78	**6.07**	0.78	**6.21**	0.79	**5.56**	0.76
2	**3.09**	0.59	**2.89**	0.46	**3.15***	0.47	**2.83**	0.58
3	**4.17**	0.77	**3.81**	0.51	**3.92**	0.44	**4.06**	0.81
4	**4.51**	0.73	**4.80**	0.60	**5.32**	0.63	**3.99**	0.66
5	**5.10**	0.78	**5.09**	0.62	**5.45**	0.69	**4.74**	0.71

The data are expressed as means (±SEM) and based on 16 observations/treatment group.
There are no significant differences between AA and non-AA cows throughout the trial.
*Daily quarter milk production in H cows tends to be higher than in non-H cows ($P < 0.090$).

Table 9 Change of Mean Daily Quarter Milk Production as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Change of Mean Daily Quarter Milk Production (%)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Day)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
1	**0.00**	0.00	**0.00**	0.00	**0.00**	0.00	**0.00**	0.00
2	**-44.40**	5.17	**-53.09**	4.07	**-46.10***	4.76	**-51.39**	4.71
3	**-27.88**	5.67	**-35.86**	6.12	**-31.95**	5.41	**-31.79**	6.51
4	**-20.29**	7.02	**-19.43**	5.00	**-12.91**	5.80	**-26.81**	5.85
5	**-14.15**	6.11	**-14.34**	4.57	**-13.64**	6.05	**-14.85**	4.66

The data are expressed as means (±SEM) and based on 16 observations/treatment group.
There are no significant differences between AA and non-AA cows throughout the trial.
* Change of mean daily quarter milk production in H cows tends to be higher than in non-H cows ($P < 0.090$).

Table 10 Mean Composite Milk Production by Hours Following LPS Challenge in Four Different Treatments; OO: LPS alone, CO: LPS + Ascorbic acid, OH: LPS + Histidine, and CH: LPS + Ascorbic acid + Histidine in dairy cattle.

Mean Composite Milk Production (kg)								
Time	**OO**		**CO**		**OH**		**CH**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**11.23**	1.69	**11.48**	1.44	**9.93**	1.39	**10.13**	1.32
0	**12.40**	2.79	**12.73**	1.78	**12.45**	1.82	**12.35**	1.62
12	**6.10**	1.20	**7.29**	1.36	**6.63**	1.31	**7.26**	1.34
24	**8.61**	1.48	**9.34**	1.64	**7.96**	0.85	**8.50**	0.93
36	**8.98**	1.30	**8.80**	1.00	**8.41**	1.08	**8.43**	1.11
48	**11.81**	1.50	**12.54***	1.45	**10.76**	1.48	**12.88****	1.24
60	**9.73**	1.63	**9.87***	1.36	**9.14**	1.29	**9.65****	1.28
72	**12.40**	1.76	**12.66***	1.72	**11.10**	1.19	**12.74****	1.52
84	**9.12**	1.69	**9.71***	1.51	**8.75**	1.50	**10.54****	1.22
96	**12.05**	1.80	**13.38***	1.83	**11.14**	1.37	**12.99****	1.26

The data are expressed as means (±SEM) and based on 8 observations/treatment group.

* Mean composite milk production in CO is significantly higher than in OH ($P = 0.0172$).

** Mean composite milk production in CH is significantly higher than in OH ($P = 0.0100$).

Table 11 Mean Composite Milk Production as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Mean Composite Milk Production (kg)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**10.81**	0.96	**10.58**	1.07	**10.03**	0.92	**11.36**	1.07
0	**12.54**	1.16	**12.42**	1.61	**12.40**	1.18	**12.56**	1.60
12	**7.28**	0.92	**6.36**	0.86	**6.94**	0.91	**6.70**	0.89
24	**8.92**	0.92	**8.29**	0.83	**8.23**	0.61	**8.98**	1.07
36	**8.61**	0.72	**8.69**	0.82	**8.42**	0.75	**8.89**	0.79
48	**12.71***	0.92	**11.29**	1.03	**11.82**	0.97	**12.17**	1.01
60	**9.76***	0.90	**9.43**	1.01	**9.39**	0.88	**9.80**	1.03
72	**12.70***	1.11	**11.75**	1.04	**11.92**	0.95	**12.53**	1.19
84	**10.13***	0.94	**8.93**	1.09	**9.65**	0.96	**9.41**	1.10
96	**13.18***	1.07	**11.59**	1.10	**12.06**	0.93	**12.71**	1.25

The data are expressed as means (±SEM) and based on 16 observations/treatment group.
* Mean Composite Milk Production in AA cows is significantly higher than in non-AA cows ($P < 0.02$). There are no significant differences between H and non-H.

Table 12 Mean Daily Composite Milk Production as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Mean Composite Milk Production (kg)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Day)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
1	**23.34**	1.92	**23.00**	2.41	**22.43**	2.05	**23.92**	2.28
2	**16.20**	1.73	**14.65**	1.62	**15.17**	1.48	**15.67**	1.88
3	**21.32***	1.55	**19.98**	1.80	**20.24**	1.60	**21.06**	1.76
4	**22.46***	1.97	**21.18**	2.00	**21.31**	1.77	**22.33**	2.18
5	**23.31***	1.96	**20.52**	2.14	**21.71**	1.82	**22.13**	2.31

The data are expressed as means (±SEM) and based on 16 observations/treatment group.
* Mean daily composite milk production in AA cows is significantly higher than in non-AA cows ($P < 0.02$). There are no significant differences between H and non-H cows.

Table 13 Change of Mean Change of Daily Composite Milk Production as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Change of Mean Daily Composite Milk Production (%)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Day)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
1	**0.00**	0.00	**0.00**	0.00	**0.00**	0.00	**0.00**	0.00
2	**-31.55**	3.32	**-35.54**	3.90	**-31.85**	3.37	**-35.24**	3.87
3	**-6.63**	3.51	**-10.54**	4.34	**-7.83**	3.97	**-9.34**	3.98
4	**-2.48***	4.37	**-4.43**	5.56	**-2.68**	4.48	**-4.23**	5.49
5	**1.77***	5.81	**-8.73**	4.06	**0.04**	6.19	**-7.01**	3.73

The data are expressed as means (±SEM) and based on 16 observations/treatment group.
* Mean daily composite milk production in AA cows is significantly higher than in non-AA cows ($P < 0.02$). There are no significant differences between H and non-H cows.

Table 14 Mean Milk IgG_1 by Hours Following LPS Challenge in Four Different Treatments; OO: LPS alone, CO: LPS + Ascorbic acid, OH: LPS + Histidine, and CH: LPS + Ascorbic acid + Histidine in dairy cattle.

Mean Milk IgG_1 (mg/ml)								
Time	**OO**		**CO**		**OH**		**CH**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
0	**0.52**	0.12	**0.40**	0.04	**0.57**	0.17	**0.45**	0.03
2	**1.40**	0.38	**2.32**	1.78	**1.80**	0.45	**0.96**	0.26
3	**1.46**	0.40	**2.14**	0.92	**1.24**	0.37	**0.86**	0.15
4	**1.93**	0.44	**3.17**	1.72	**1.26***	0.47	**0.95****	0.18
6	**2.19**	0.72	**5.45**	2.44	**1.36***	0.36	**1.30****	0.37
9	**2.85**	1.02	**2.18**	0.94	**1.25***	0.56	**1.04****	0.24
12	**1.85**	0.75	**3.11**	1.39	**2.02***	0.40	**1.62****	0.21
24	**1.21**	0.32	**3.46**	1.75	**1.11***	0.15	**1.42**	0.33
36	**0.94**	0.28	**1.61**	0.74	**0.82***	0.12	**0.92**	0.25
48	**0.83**	0.29	**0.84**	0.23	**0.68**	0.11	**0.69**	0.10

The data are expressed as means (±SEM) and based on 8 observations/treatment group.

* Mean milk IgG_1 in CO tends to be higher than in OH ($P < 0.08$).

** Mean milk IgG_1 in CO is significantly higher than CH ($P < 0.05$).

Table 15 Mean Milk IgG_1 as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Mean Milk IgG_1 (mg/ml)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
0	**0.42**	0.03	**0.54**	0.10	**0.51**	0.09	**0.46**	0.06
2	**1.64**	0.89	**1.60**	0.29	**1.38**	0.27	**1.86**	0.89
3	**1.50**	0.48	**1.35**	0.27	**1.05****	0.20	**1.80**	0.49
4	**2.06**	0.88	**1.59**	0.32	**1.11****	0.25	**2.55**	0.87
6	**3.38***	1.31	**1.78**	0.40	**1.33****	0.25	**3.82**	1.30
9	**1.61**	0.49	**2.05**	0.60	**1.14****	0.29	**2.52**	0.67
12	**2.36**	0.71	**1.93**	0.41	**1.82**	0.22	**2.48**	0.78
24	**2.44***	0.90	**1.16**	0.17	**1.27**	0.18	**2.33**	0.91
36	**1.26**	0.39	**0.88**	0.15	**0.87**	0.13	**1.28**	0.39
48	**0.76**	0.12	**0.76**	0.15	**0.69**	0.07	**0.83**	0.18

The data are expressed as means (±SEM) and based on 16 observations/treatment group.

* Mean milk IgG_1 in AA cows tends to be higher than in non-AA cows ($P < 0.10$).

** Mean milk IgG_1 in H cows tends to be lower than in non-H cows ($P = 0.0555$).

Table 16 Mean Antioxidant Activities (AOA) as % Inhibition by Hours Following LPS Challenge in Four Different Treatments; OO: LPS alone, CO: LPS + Ascorbic acid, OH: LPS + Histidine, and CH: LPS + Ascorbic acid + Histidine in dairy cattle.

Mean Antioxidant Activities (% Inhibition)								
Time	**OO**		**CO**		**OH**		**CH**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
0	**62.14**	2.51	**66.60**	4.02	**61.33**	2.06	**67.25**	3.44
2	**63.01**	3.86	**61.33**	2.11	**60.38**	3.04	**62.91**	2.79
3	**63.72**	3.59	**64.17**	4.37	**61.60**	3.00	**62.94**	1.73
4	**62.51**	3.00	**65.56**	3.23	**61.55**	2.21	**60.74**	2.93
6	**66.78**	3.56	**71.64***	2.73	**63.30***	2.86	**62.60***	2.75
9	**63.18**	2.26	**66.20***	3.42	**57.67***	3.95	**59.52***	2.98
12	**64.91**	2.63	**65.22***	3.07	**60.35***	3.18	**61.28***	2.12

The data are expressed as means (±SEM) and based on 8 observations/treatment group.
* Mean AOA in CO is significantly higher than in OH and CH ($P < 0.03$).

Table 17 Mean Antioxidant Activities (AOA, % inhibition) as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Mean Antioxidant Activities (% Inhibition)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
0	**66.93**	2.53	**61.73**	1.57	**64.29**	2.05	**64.37**	2.36
2	**62.62**	1.69	**61.70**	2.40	**61.65**	2.02	**62.67**	2.13
3	**63.55**	2.28	**62.73**	2.29	**62.31**	1.65	**63.94**	2.73
4	**63.15**	2.20	**62.03**	1.81	**61.14**	1.78	**64.03**	2.16
6	**67.12**	2.21	**65.04**	2.25	**62.95***	1.92	**69.21**	2.26
9	**62.86**	2.36	**60.42**	2.31	**58.59***	2.40	**64.69**	2.02
12	**63.25**	1.87	**62.63**	2.08	**60.82***	1.85	**65.07**	1.95

The data are expressed as means (±SEM) and based on 16 observations/treatment group.
No significant difference of mean AOA between AA and non-AA cows.
* Mean AOA in H cows is significantly lower than in non-H cows ($P < 0.04$).

Table 18 Mean Plasma Ascorbic Acid concentration by Hours Following LPS Challenge in CO (n = 8): LPS + Ascorbic acid, and CH (n = 8): LPS + Ascorbic acid + Histidine in dairy cattle.

Mean Plasma Ascorbic Acid (μM/L)				
Time (Hour)	CO		CH	
	Mean	SEM	Mean	SEM
0	**11.28**	1.80	**12.27**	2.61
2	**14.00**	2.28	**14.72**	2.79
3	**11.85**	1.94	**12.24**	2.54
4	**423.08**	87.83	**433.81**	98.68
6	**626.79**	96.96	**681.12**	98.42
9	**101.88**	15.34	**103.32**	12.00
12	**63.09**	6.84	**60.47**	6.29
24	**36.09**	3.07	**33.10**	4.49

The data are expressed as means (±SEM) and based on 8 observations/treatment group.
No significant difference of mean ascorbic acid concentration between CO and CH.

Table 19 Mean Heart Rate by Hours Following LPS Challenge in Four Different Treatments; OO: LPS alone, CO: LPS + Ascorbic acid, OH: LPS + Histidine, and CH: LPS + Ascorbic acid + Histidine in dairy cattle.

Mean Heart Rate (per minute)								
Time	**OO**		**CO**		**OH**		**CH**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**80.00**	2.86	**83.75**	3.39	**83.25**	3.00	**81.86**	3.50
0	**75.17**	3.47	**77.88**	3.53	**81.13**	6.08	**73.71**	3.23
2	**88.00**	7.36	**84.63**	3.21	**89.13**	7.74	**81.29**	3.63
3	**92.17**	3.52	**99.63**	3.15	**98.38**	3.42	**96.43**	4.90
4	**91.00**	3.60	**105.00**	3.70	**98.75**	6.22	**101.86**	4.58
6	**97.00**	4.77	**104.50**	3.83	**103.50**	7.49	**102.86**	4.28
9	**83.50**	2.64	**93.13**	2.52	**84.25**	5.19	**89.43**	2.38
12	**75.83**	3.62	**79.00**	2.65	**84.75**	2.50	**77.14**	4.53
24	**76.33**	3.99	**79.75**	2.74	**81.50**	2.77	**82.86**	3.87
36	**78.17**	2.01	**83.75**	1.62	**79.13**	3.89	**85.57**	2.80
48	**74.83**	3.76	**75.63**	3.09	**75.88**	4.08	**75.43**	2.57

The data are expressed as means (±SEM) and based on 8 observations/treatment group.
No significant difference among four treatments throughout the experimental period.

Table 20 Mean Heart Rate as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Mean Heart Rate (per minute)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**82.56**	2.30	**81.88**	1.91	**82.47**	2.23	**82.13**	2.07
0	**75.88**	2.29	**80.00**	3.52	**77.60**	3.52	**78.38**	2.57
2	**82.88**	2.29	**88.06**	4.82	**85.20**	4.35	**85.81**	3.42
3	**98.25**	2.70	**96.00**	2.65	**97.20**	2.82	**96.63**	2.61
4	**103.81**	2.76	**97.50**	3.82	**101.40**	3.75	**100.63**	3.15
6	**103.50**	2.68	**101.88**	4.35	**103.73**	4.17	**102.38**	3.07
9	**91.25***	1.69	**84.94**	2.84	**87.80**	2.72	**89.38**	2.04
12	**78.63**	2.45	**81.69**	2.26	**82.07**	2.58	**78.81**	2.16
24	**81.38**	2.22	**80.13**	2.28	**81.73**	2.21	**79.25**	2.24
36	**85.06**	1.56	**79.25**	2.10	**83.47**	2.45	**81.56**	1.35
48	**75.81**	1.91	**75.31**	2.57	**75.93**	2.38	**75.19**	2.23

The data are expressed as means (±SEM) and based on 16 observations/treatment group.
* Mean heart rate in AA cows tends to be higher than in non-AA cows ($P < 0.10$).
No significant difference between H and non-H.

Table 21 Mean Respiratory Rate by Hours Following LPS Challenge in Four Different Treatments; OO: LPS alone, CO: LPS + Ascorbic acid, OH: LPS + Histidine, and CH: LPS + Ascorbic acid + Histidine in dairy cattle.

Mean Respiratory Rate (per minute)								
Time	**OO**		**CO**		**OH**		**CH**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**36.25**	4.63	**36.50**	5.32	**35.00**	4.60	**27.50**	2.34
0	**39.38**	4.48	**35.13**	3.32	**37.00**	2.91	**32.57**	3.51
2	**37.25**	5.15	**43.13**	7.25	**35.14**	3.78	**35.57**	4.70
3	**36.00**	2.65	**38.00**	4.84	**38.29**	5.60	**35.71**	4.22
4	**36.75**	3.85	**33.00**	3.21	**40.29**	9.38	**31.57**	3.77
6	**52.38**	10.38	**43.13**	8.42	**50.71**	11.36	**52.29**	11.86
9	**44.25**	6.96	**42.63**	8.17	**36.14**	6.52	**34.00**	5.47
12	**35.88**	6.16	**34.88***	4.86	**41.29**	7.56	**33.00**	5.08
24	**30.00**	3.09	**28.50***	1.40	**33.43**	3.36	**33.71**	3.40
36	**37.50**	5.49	**32.25***	2.78	**36.86**	5.01	**34.57**	4.98
48	**37.00**	4.66	**34.75***	2.67	**35.43**	3.43	**34.86**	3.41

The data are expressed as means (±SEM) and based on 8 observations/treatment group.
* Mean respiratory rate in CO tends to be lower than in OH ($P < 0.10$).

Table 22 Mean Respiratory Rate (per minute) as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Mean Respiratory Rate (per minute)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**33.69**	3.19	**37.31**	3.46	**32.80**	2.84	**36.38**	3.41
0	**33.75**	2.12	**38.88**	2.61	**34.53**	2.03	**37.25**	2.75
2	**39.00**	4.09	**36.00**	3.03	**35.00**	2.58	**40.19**	4.36
3	**36.38**	2.91	**36.75**	2.85	**36.40**	3.15	**37.00**	2.68
4	**32.56**	2.13	**38.00**	4.63	**35.93**	4.72	**34.88**	2.47
6	**46.69**	6.23	**51.63**	7.14	**50.47**	7.11	**47.75**	6.56
9	**40.06**	4.82	**39.69**	4.63	**36.87**	4.11	**43.44**	5.19
12	**33.94**	3.05	**38.38**	4.55	**36.87**	4.17	**35.38**	3.79
24	**30.88**	1.60	**32.25**	2.26	**33.33**	2.07	**29.25**	1.65
36	**33.25**	2.35	**37.63**	3.50	**35.47**	3.07	**34.88**	3.05
48	**34.50**	1.86	**36.00**	2.74	**34.80**	2.11	**35.88**	2.61

The data are expressed as means (±SEM) and based on 16 observations/treatment group.
No significant difference between AA and non-AA cows, as well as between H and non-H cows.

Table 23 Mean Ruminal Contraction Rate by Hours Following LPS Challenge in Four Different Treatments; OO: LPS alone, CO: LPS + Ascorbic acid, OH: LPS + Histidine, and CH: LPS + Ascorbic acid + Histidine in dairy cattle.

Mean Ruminal Contraction Rate (per minute)								
Time	**OO**		**CO**		**OH**		**CH**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**1.50**	0.19	**1.63**	0.26	**1.50**	0.19	**1.29**	0.27
0	**1.33**	0.18	**1.25**	0.16	**1.38**	0.18	**1.14**	0.13
2	**1.17**	0.27	**1.00**	0.27	**0.88**	0.30	**1.14**	0.32
3	**1.00**	0.22	**1.00**	0.27	**0.75**	0.16	**1.14**	0.13
4	**0.67**	0.18	**0.63***	0.26	**0.25**	0.16	**0.43**	0.19
6	**0.50**	0.19	**0.88***	0.13	**0.38**	0.18	**0.57**	0.19
9	**1.00**	0.00	**1.13***	0.13	**0.88**	0.23	**1.00**	0.20
12	**1.33**	0.18	**1.38**	0.18	**1.25**	0.16	**1.43**	0.19
24	**1.33**	0.18	**1.38**	0.26	**1.25**	0.25	**1.29**	0.27
36	**1.17**	0.27	**1.38**	0.26	**1.38**	0.26	**1.57**	0.28
48	**1.17**	0.27	**1.13**	0.23	**1.25**	0.25	**1.14**	0.24

The data are expressed as means (±SEM) and based on 8 observations/treatment group.
* Mean ruminal contraction rate in CO is significantly higher than in OH (P = 0.0504).

Table 24 Mean Ruminal Contraction Rate (per minute) as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Mean Ruminal Contraction Rate (per minute)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Hour)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-12	**1.50**	0.18	**1.50**	0.13	**1.47**	0.16	**1.56**	0.16
0	**1.19**	0.10	**1.31**	0.12	**1.20**	0.10	**1.25**	0.11
2	**1.13**	0.20	**1.00**	0.18	**1.13**	0.21	**1.06**	0.17
3	**1.06***	0.14	**0.75**	0.14	**0.93**	0.11	**0.88**	0.18
4	**0.56***	0.16	**0.38**	0.13	**0.40**	0.13	**0.56**	0.16
6	**0.75***	0.11	**0.44**	0.13	**0.53**	0.13	**0.69**	0.12
9	**1.06***	0.11	**0.88**	0.13	**0.93**	0.15	**1.00**	0.09
12	**1.38**	0.13	**1.25**	0.11	**1.33**	0.12	**1.31**	0.12
24	**1.38**	0.18	**1.25**	0.17	**1.33**	0.18	**1.31**	0.18
36	**1.50**	0.18	**1.25**	0.19	**1.60**	0.16	**1.25**	0.19
48	**1.13**	0.15	**1.25**	0.17	**1.20**	0.17	**1.19**	0.16

The data are expressed as means (±SEM) and based on 16 observations/treatment group.
* Mean ruminal contraction rate in AA cows tends to be higher than in non-AA cows ($P < 0.10$).
No significant difference between H and non-H cows was seen.

Table 25 Mean Dry Matter Intake (lbs.) by Hours Following LPS Challenge in Four Different Treatments; OO: LPS alone, CO: LPS + Ascorbic acid, OH: LPS + Histidine, and CH: LPS + Ascorbic acid + Histidine in dairy cattle.

Mean Dry Matter Intake (lbs.)								
Time	**OO**		**CO**		**OH**		**CH**	
(Day)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-1	**38.09***	4.01	**41.95**	2.36	**40.47****	3.18	**44.18**	3.73
0	**37.17***	3.29	**37.51**	2.62	**34.85****	3.23	**41.98**	3.03
1	**36.41***	3.34	**38.32**	3.08	**35.50****	3.62	**43.83**	2.69
2	**37.07***	3.99	**39.62**	2.47	**36.23****	3.35	**44.89**	3.25
3	**39.07**	3.98	**42.59**	3.39	**37.89**	2.93	**42.30**	4.25
4	**39.36**	3.55	**44.53**	2.52	**39.31**	2.61	**43.53**	4.52
5	**40.36**	3.46	**47.24**	2.40	**41.83**	2.16	**45.24**	3.74

The data are expressed as means (±SEM) and based on 8 observations/treatment group.
*Difference between OO and CH is statistically significant ($P < 0.04$).
**Difference between OH and CH is statistically significant ($P < 0.02$).
***Difference between OO and CO approaches statistical significance ($P < 0.10$).

Table 26 Mean Dry Matter Intake (lbs.) as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Mean Dry Matter Intake (lbs.)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Day)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-1	**43.06***	2.15	**39.28**	2.49	**42.32**	2.42	**40.02**	2.30
0	**39.74***	2.02	**36.01**	2.25	**38.42**	2.33	**37.34**	2.03
1	**41.08***	2.10	**35.96**	2.38	**39.67**	2.43	**37.37**	2.21
2	**42.26***	2.09	**36.65**	2.52	**40.56**	2.52	**38.35**	2.29
3	**42.45***	2.60	**38.48**	2.39	**40.10**	2.56	**40.83**	2.54
4	**44.03***	2.50	**39.34**	2.15	**41.56**	2.62	**41.95**	2.20
5	**46.24***	2.16	**41.05**	2.01	**43.56**	2.17	**43.80**	2.22

The data are expressed as means (± SEM) and based on 16 observations/treatment group.
*Mean DMI in AA had been statistically significant higher ($P < 0.06$) than in Non-AA.
There was no significant difference between H and Non-H.

Table 27 Mean Dry Matter Intake (% Reduction) by Hours Following LPS Challenge in Four Different Treatments; OO: LPS alone, CO: LPS + Ascorbic acid, OH: LPS + Histidine, and CH: LPS + Ascorbic acid + Histidine in dairy cattle.

Mean Dry Matter Intake (%Reduction)								
Time	**OO**		**CO**		**OH**		**CH**	
(Day)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-1	**0.00**	0.00	**0.00**	0.00	**0.00**	0.00	**0.00**	0.00
0	**0.73***	6.86	**-10.77**	4.12	**-13.59**	4.89	**-2.84**	6.01
1	**-0.36***	8.46	**-9.36**	4.08	**-10.63**	7.24	**1.51**	5.43
2	**3.80**	12.83	**-5.51**	3.35	**-7.58**	8.47	**4.26**	7.06
3	**9.55**	13.11	**1.60**	5.36	**-2.81**	8.57	**-1.06**	10.56
4	**11.70**	14.66	**6.62**	3.36	**4.41**	9.26	**1.96**	11.94
5	**12.89**	12.91	**13.28**	3.31	**11.18**	9.16	**7.28**	12.79

The data are expressed as means (± SEM) and based on 8 observations/treatment group.
*Difference between OO and OH approaches statistical significance ($P < 0.10$).

Table 28 Mean Dry Matter Intake (% Reduction) as Treatment Groups; AA: CO + CH, H: OH + CH, Non-AA: OO + OH, Non-H: OO + CO, by Hours Following LPS Challenge in Four Different Treatments; OO (LPS alone), CO (LPS + Ascorbic acid), OH (LPS + Histidine), and CH (LPS + Ascorbic acid + Histidine) in dairy cattle.

Mean Dry Matter Intake (% Reduction)								
Time	**AA**		**Non-AA**		**H**		**Non-H**	
(Day)	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
-1	**0.00**	0.00	**0.00**	0.00	**0.00**	0.00	**0.00**	0.00
0	**-6.80**	3.67	**-8.22**	3.99	**-6.43**	4.47	**-5.02**	4.14
1	**-3.93**	3.57	**-4.56**	4.64	**-5.50**	5.54	**-4.86**	4.68
2	**-0.62**	3.98	**-1.66**	5.54	**-1.89**	7.57	**-0.86**	6.52
3	**0.27**	5.73	**-1.93**	6.57	**3.37**	7.73	**5.57**	6.92
4	**4.29**	6.02	**3.10**	7.36	**8.30**	8.54	**9.16**	7.29
5	**10.28**	6.43	**9.10**	7.69	**12.09**	7.73	**13.09**	6.44

The data are expressed as means (±SEM) and based on 16 observations/treatment group.
No significant difference between AA and Non-AA, also between H and Non-H.

APPENDIX B

Table 29 Experimental Design: Latin Square Cross-over Design

Cow	Period 1	Period 2	Period 3	Period 4
1	OO	CO	OH	CH
2	CO	OH	CH	OO
3	OH	CH	OO	CO
4	CH	OO	CO	OH
	LR	RF	RR	LF
5	OO	CO	OH	CH
6	CO	OO	CH	OH
7	OH	CH	OO	CO
8	CH	OH	CO	OO
	RR	LF	RF	LR
2937	**CO**	**OO**	**CH**	**OH**
3049	**OH**	**CO**	**OO**	**CH**
2952	**CH**	**OH**	**CO**	**OO**
2612	**OO**	**CH**	**OH**	**CO**
	LF	**RR**	**LR**	**RF**
3268	**OO**	**CO**	**OH**	**CH**
3133	**CO**	**CH**	**OO**	**OH**
2926	**OH**	**OO**	**CH**	**CO**
2813	**CH**	**OH**	**CO**	**OO**
	RF	**LR**	**LF**	**RR**

Table 30 Data Collecting Schedule

	Time (hour)														
	-12	0	2	3	4	6	9	12	24	36	48	60	72	96	1 wk
TPR/ RC*															
SCC															
Milk IgG_1															
Plasma															
Milk Weight															
Culture															

* Temperature, Pulse, Respiratory, and Ruminal contraction

LPS **AA/H** **AA/H**

APPENDIX C

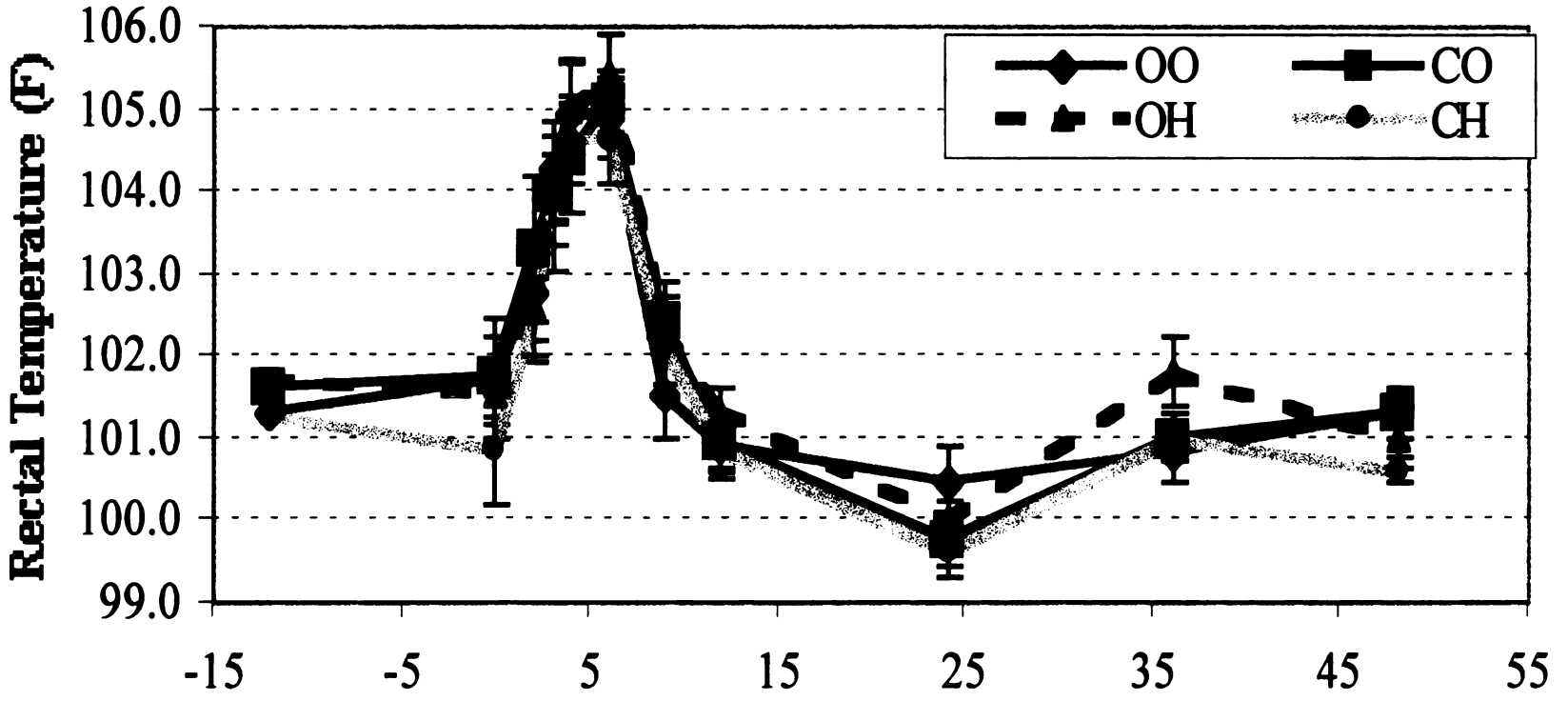

Figure 21 Mean Rectal Temperature by Hours Following LPS Challenge in Four Different Treatments; OO (n = 8): LPS alone, CO (n = 8): LPS + Ascorbic acid, OH (n = 8): LPS + L-histidine, and CH (n = 8): LPS + Ascorbic acid + L-histidine in dairy **cattle**. Difference between CO and CH approaches statistical significance ($P < 0.10$) at 48 hours post-LPD challenge. Mean rectal temperature in CO tended to be higher than in CH from at challenge and 2 hour post LPS challenge ($P < 0.10$). Mean rectal temperature in OH is significantly higher than in all other treatments ($P < 0.040$) at 36 hours post LPS challenge. Mean rectal temperature in OO is significantly higher than in CO and CH at 24 and 48 hours post challenge ($P < 0.050$).

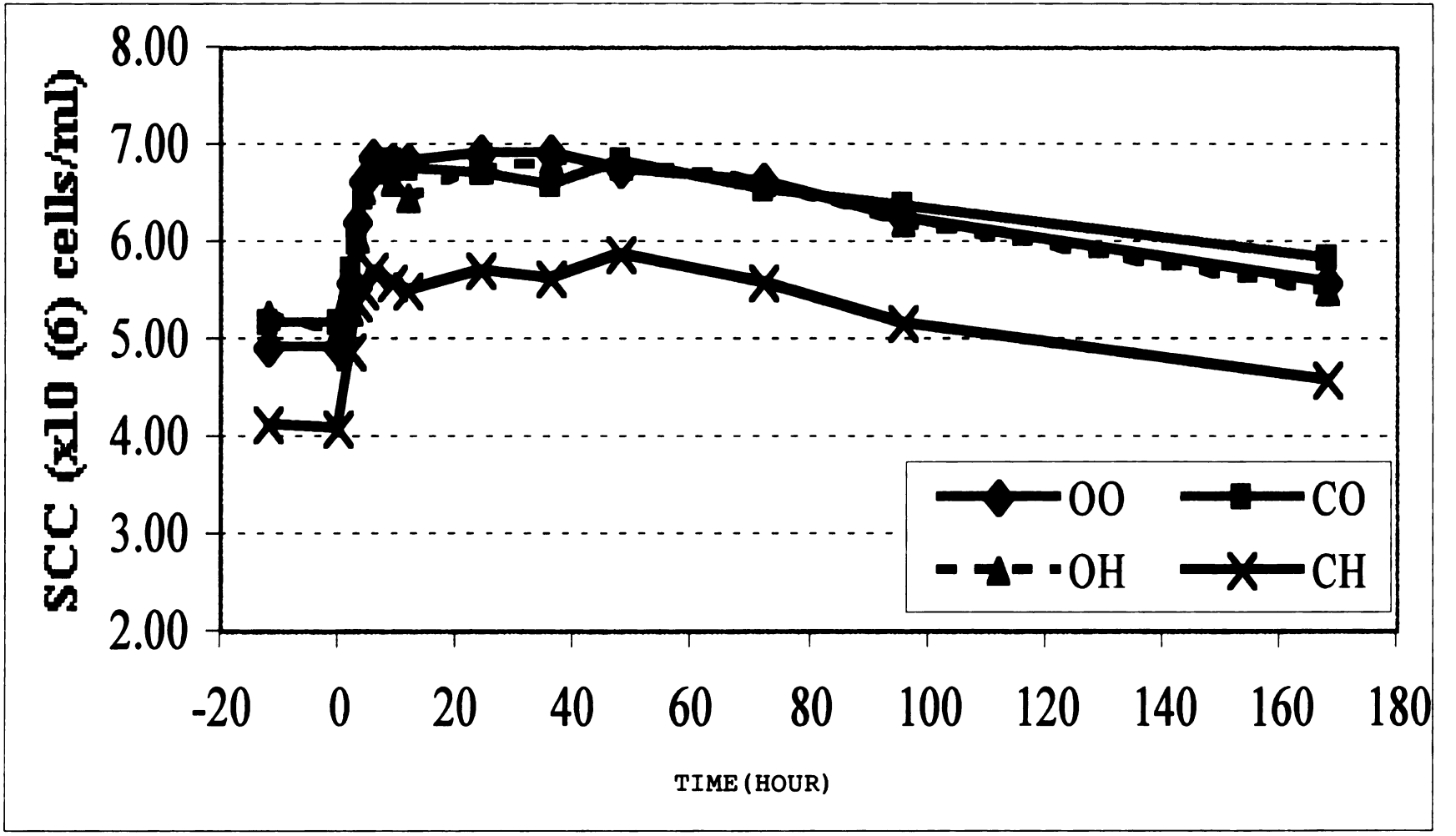

Figure 22 Mean Somatic Cell Count (SCC) by Hours Following LPS Challenge in Four Different Treatments; OO (n = 8): LPS alone, CO (n = 8): LPS + Ascorbic acid, OH (n = 8): LPS + L-histidine, and CH (n = 8): LPS + Ascorbic acid + L-histidine in dairy cattle. Mean SCC in OO is significantly higher than in CO ($P = 0.0222$), in OH ($P = 0.0255$), and in CH ($P = 0.0058$).

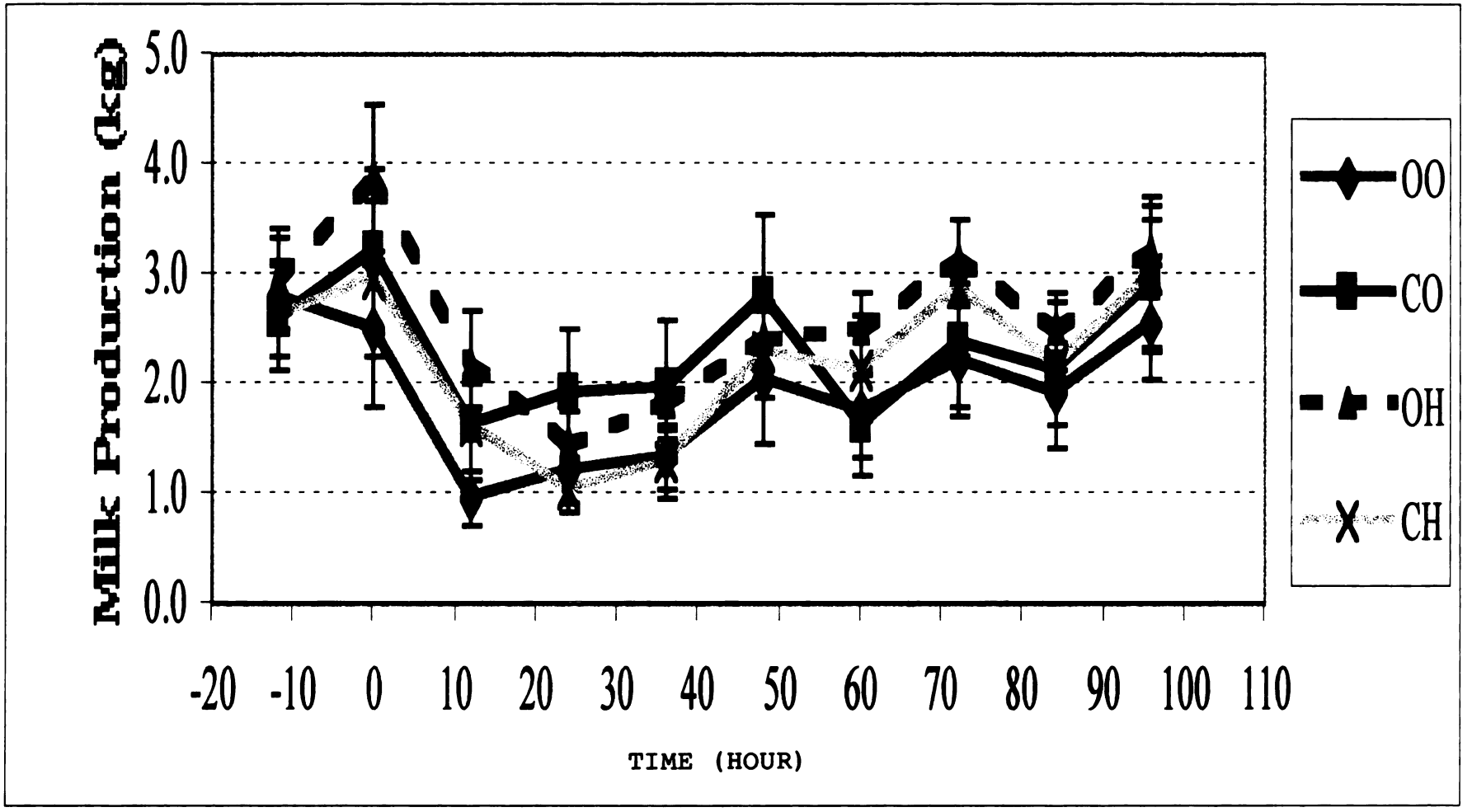

Figure 23 Mean Quarter Milk Production by Hours Following LPS Challenge in Four Different Treatments; OO (n = 8): LPS alone, CO (n = 8): LPS + Ascorbic acid, OH (n = 8): LPS + L-histidine, and CH (n = 8): LPS + Ascorbic acid + L-histidine in dairy cattle. Differences among treatments were not significant throughout the experiment.

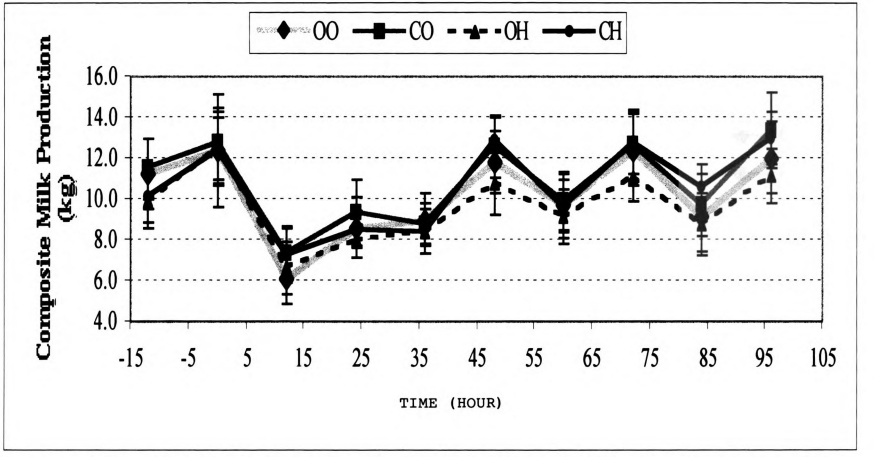

Figure 24 Mean Composite Milk Production by Hours Following LPS Challenge in Four Different Treatments; OO (n = 8): LPS alone, CO (n = 8): LPS + Ascorbic acid, OH (n = 8): LPS + L-histidine, and CH (n = 8): LPS + Ascorbic acid + L-histidine in dairy cattle. Mean composite milk production in CO is significantly higher than in OH from 48 to 96 hours post-LPS challenge (P = 0.0172). Mean composite milk production in CH is significantly higher than in OH from 48 to 96 hours post-LPS challenge (P = 0.0100).

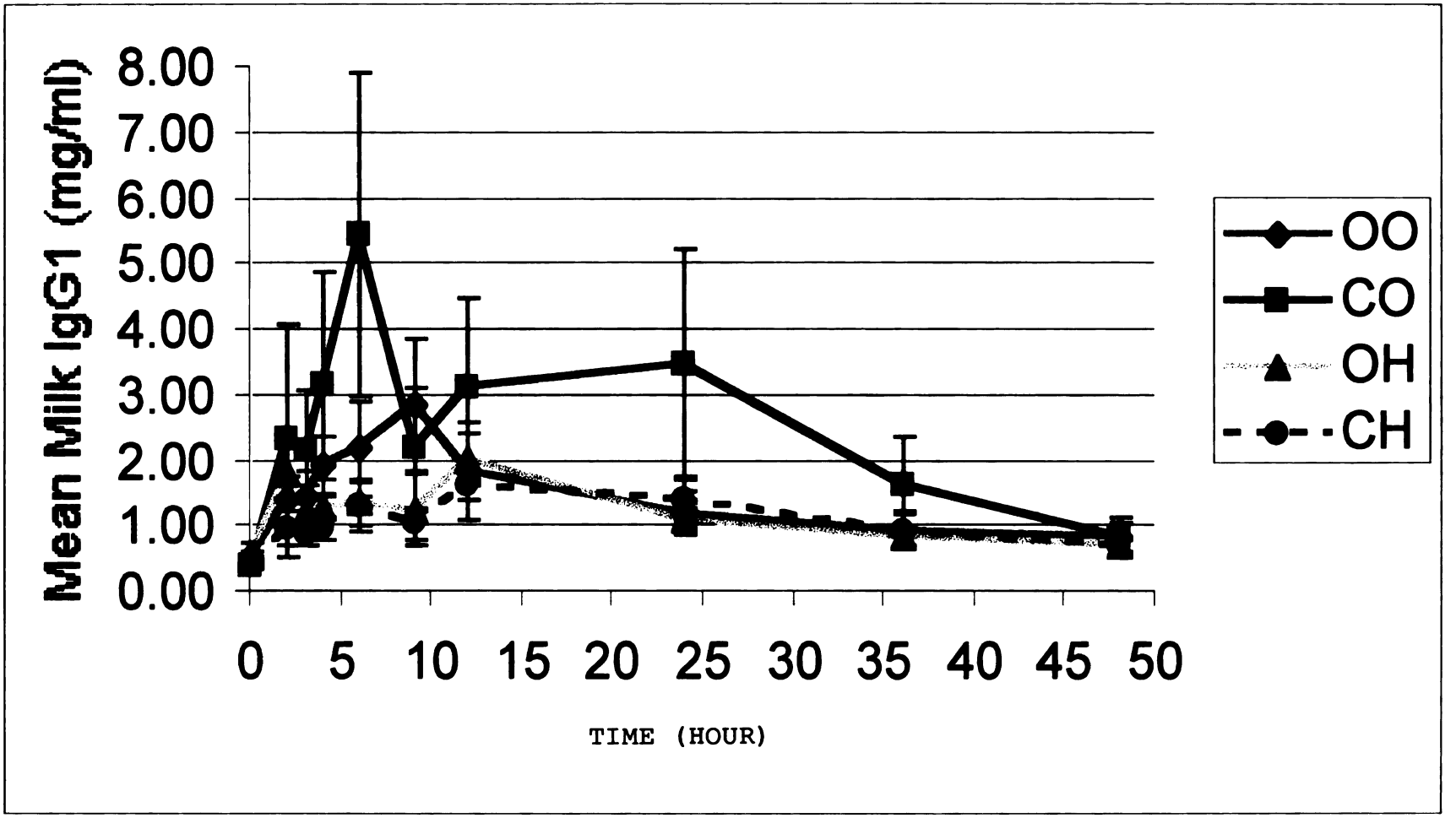

Figure 25 Mean Milk IgG_1 by Hours Following LPS Challenge in Four Different Treatments; OO (n = 8): LPS alone, CO (n = 8): LPS + Ascorbic acid, OH (n = 8): LPS + L-histidine, and CH (n = 8): LPS + Ascorbic acid + L-histidine in dairy cattle. Mean milk IgG_1 in CO tended to be higher than in OH from4 to 36 hours post-LPS challenge ($P < 0.08$). Mean milk IgG_1 in CO is significantly higher than CH from 4 to 12 hours after challenge ($P < 0.05$).

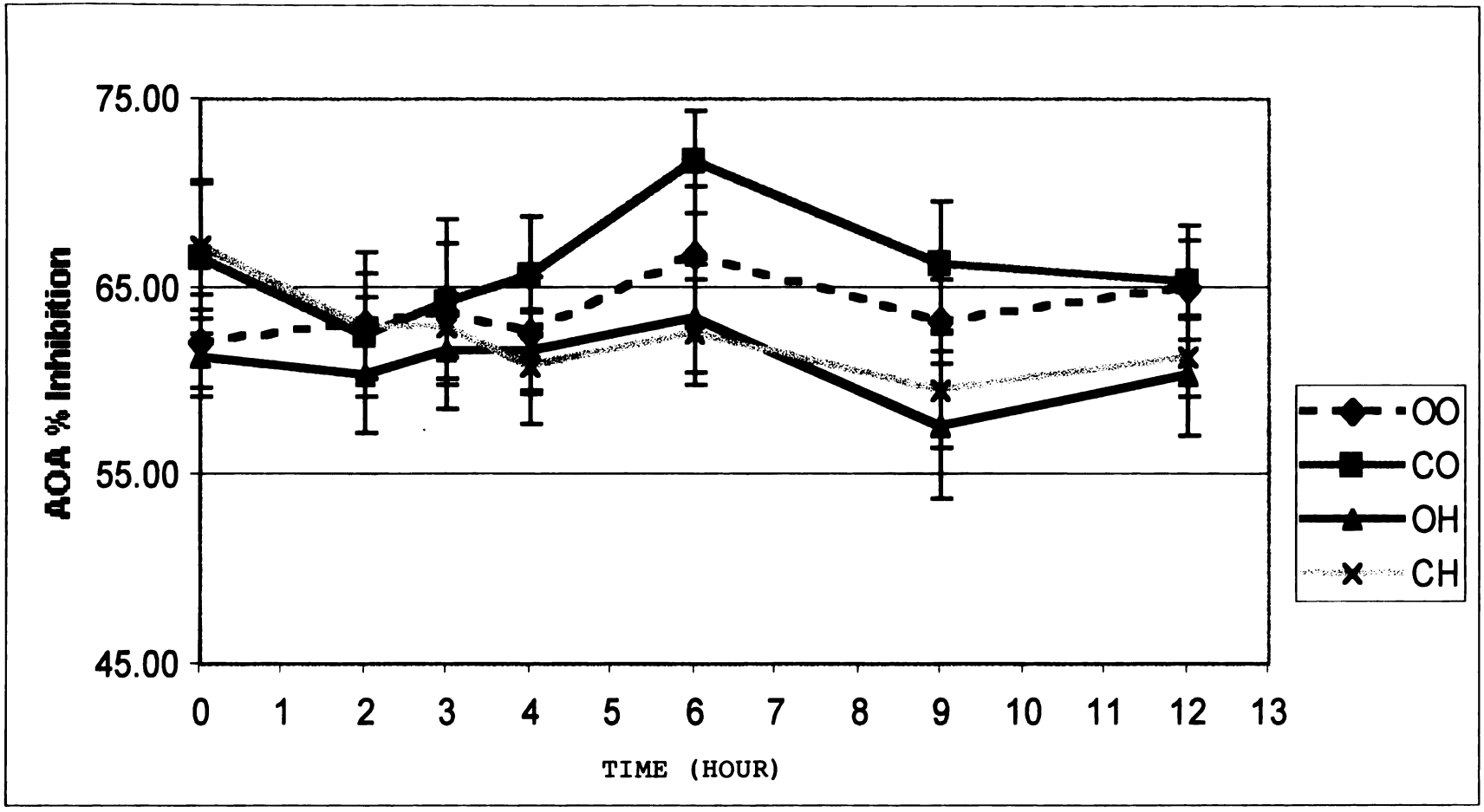

Figure 26 Mean Antioxidant Activities (AOA) as % Inhibition by Hours Following LPS Challenge in Four Different Treatments; OO (n = 8): LPS alone, CO (n = 8): LPS + Ascorbic acid, OH (n = 8): LPS + L-histidine, and CH (n = 8): LPS + Ascorbic acid + L-histidine in dairy cattle. Mean AOA in CO is significantly higher than in OH and CH at 6 hours post-LPS challenge ($P < 0.03$). There were no significant differences of mean AOA in OO and other groups throughout the trial.

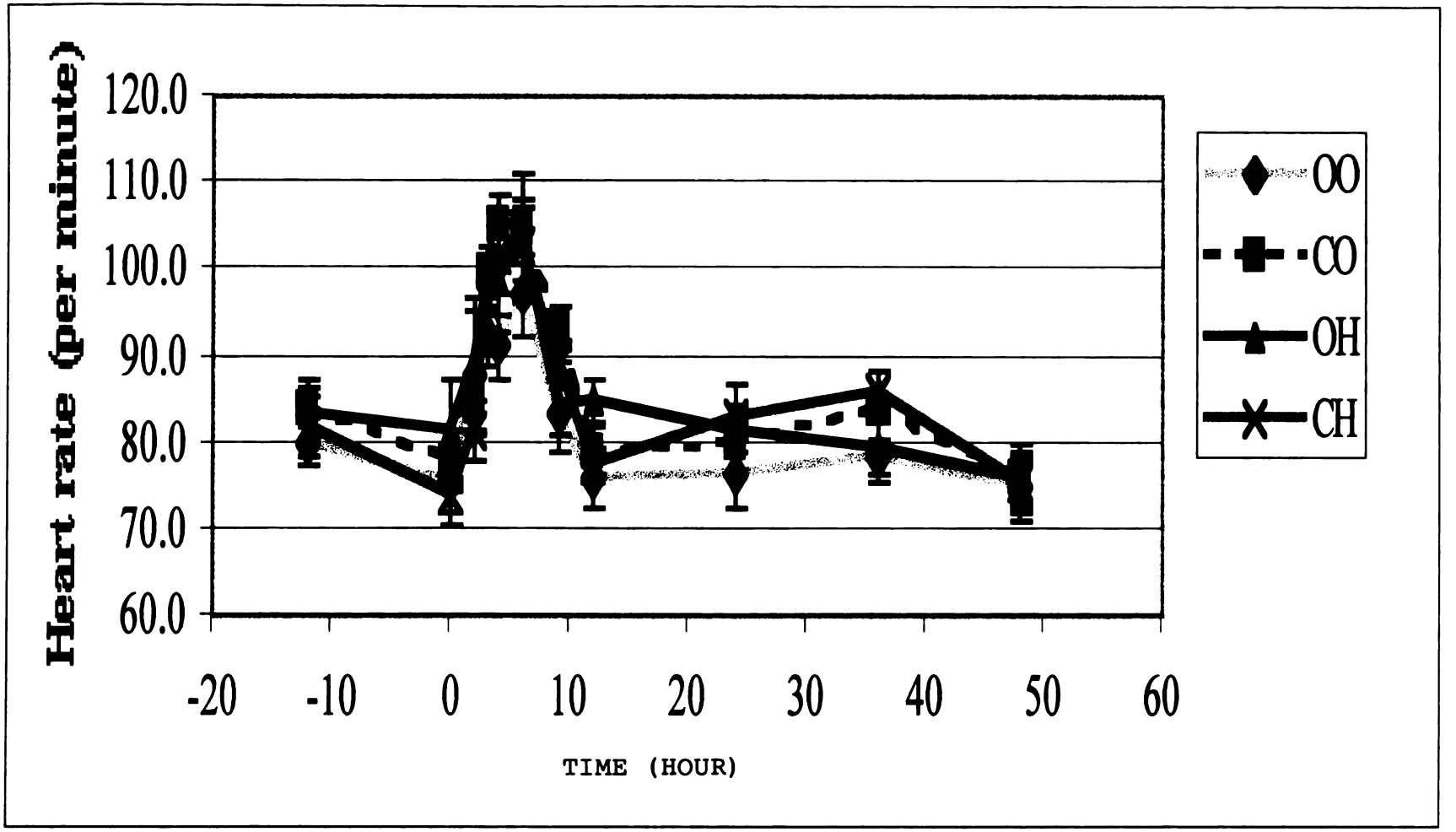

Figure 27 Mean Heart Rate by Hours Following LPS Challenge in Four Different Treatments; OO (n = 8): LPS alone, CO (n = 8): LPS + Ascorbic acid, OH (n = 8): LPS + L-histidine, and CH (n = 8): LPS + Ascorbic acid + L-histidine in dairy cattle. There were no significant differences among four treatments throughout the experimental period.

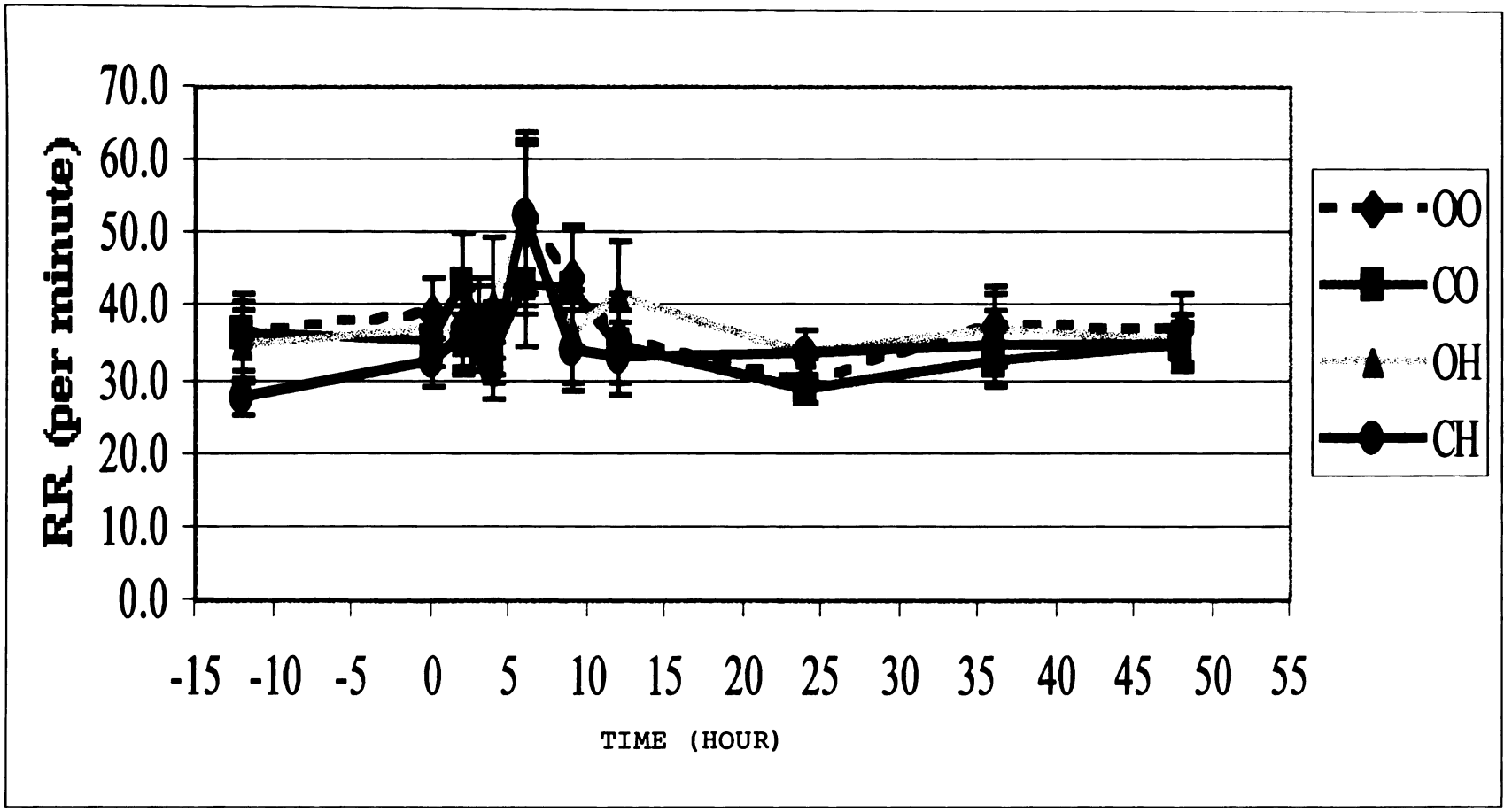

Figure 28 Mean Respiratory Rate by Hours Following LPS Challenge in Four Different Treatments; OO (n = 8): LPS alone, CO (n = 8): LPS + Ascorbic acid, OH (n = 8): LPS + L-histidine, and CH (n = 8): LPS + Ascorbic acid + L-histidine in dairy cattle. Mean respiratory rate in CO tended to be lower than in OH from 12 to 36 hours post-LPS challenge ($P < 0.10$).

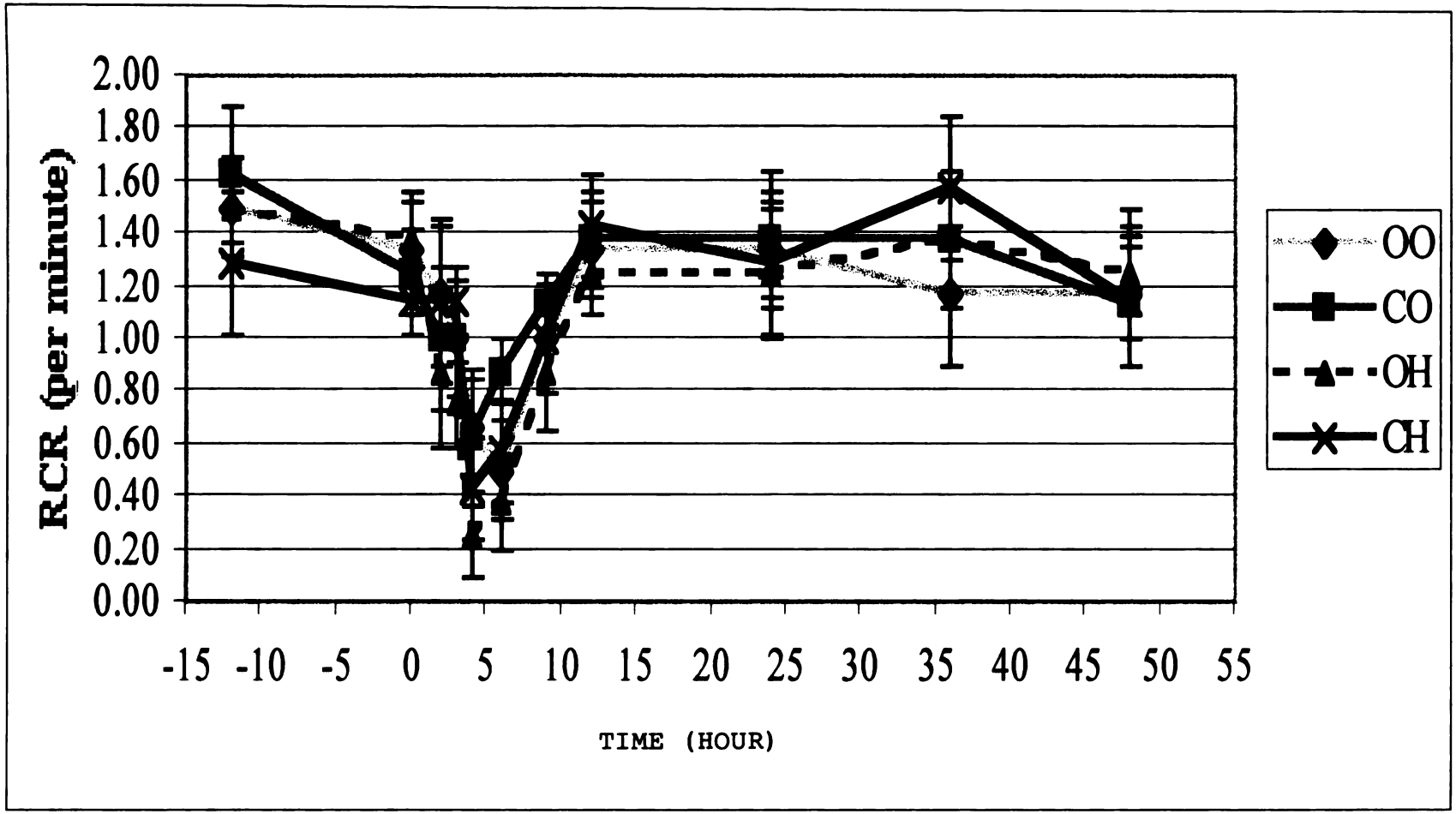

Figure 29 Mean Ruminal Contraction Rate by Hours Following LPS Challenge in Four Different Treatments; OO (n = 8): LPS alone, CO (n = 8): LPS + Ascorbic acid, OH (n = 8): LPS + L-histidine, and CH (n = 8): LPS + Ascorbic acid + L-histidine in dairy cattle. Mean ruminal contraction rate in CO is significantly higher than in OH from 4 to 6 hours post-LPS challenge (P = 0.0504).

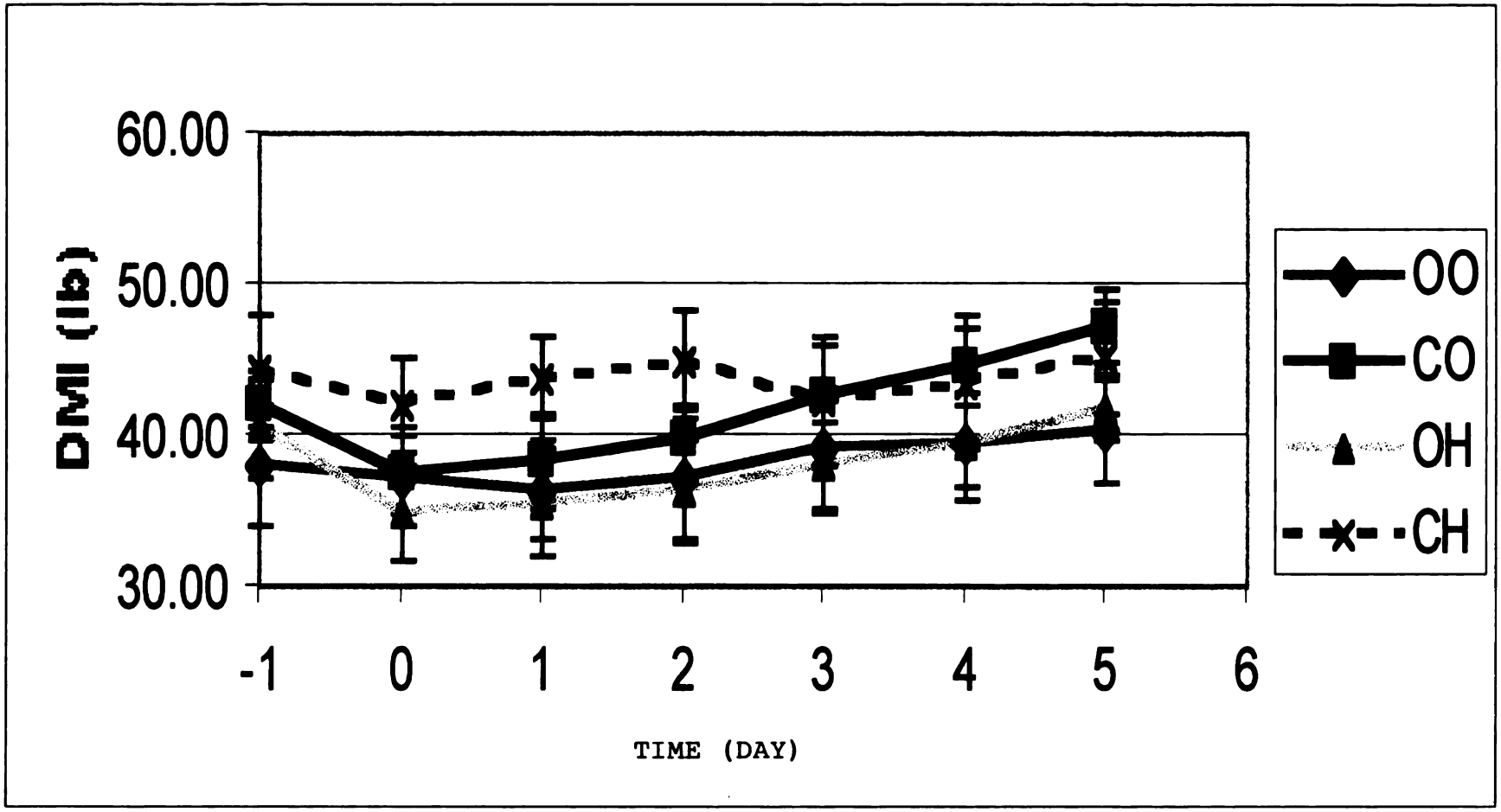

Figure 30 Mean Dry Matter Intake (lbs.) by Hours Following LPS Challenge in Four Different Treatments; OO (n = 8): LPS alone, CO (n = 8): LPS + Ascorbic acid, OH (n = 8): LPS + L-histidine, and CH (n = 8): LPS + Ascorbic acid + L-histidine in dairy cattle. Difference between OO and CH is statistically significant ($P < 0.04$) from D(-1) to D2. Difference between OH and CH is also statistically significant ($P < 0.02$) from D(-1) to D2. Difference between OO and CO group approaches statistical significance ($P < 0.10$) from D3 to D5.

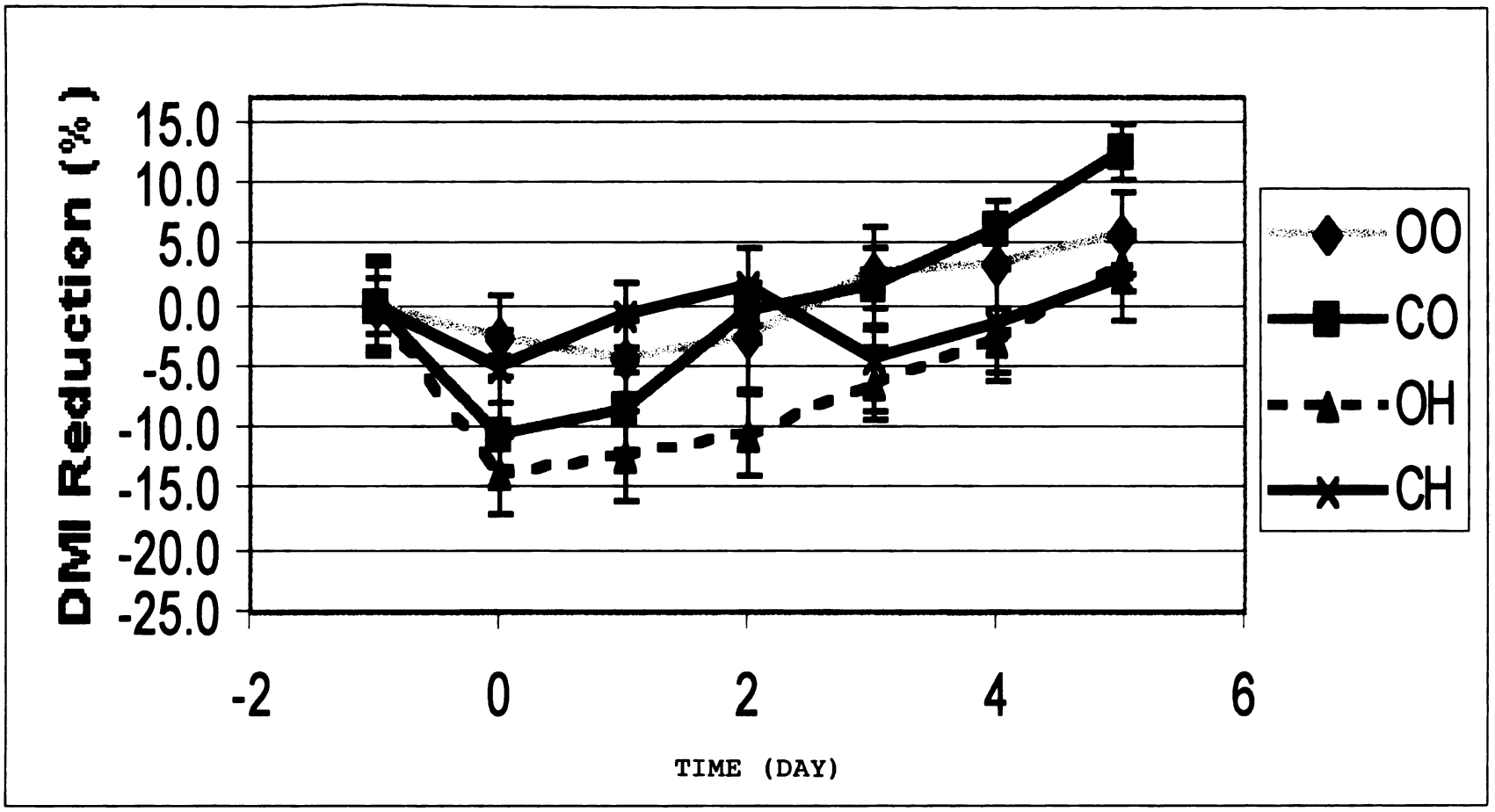

Figure 31 Mean Dry Matter Intake (% Reduction) by Hours Following LPS Challenge in Four Different Treatments; OO (n = 8): LPS alone, CO (n = 8): LPS + Ascorbic acid, OH (n = 8): LPS + L-histidine, and CH (n = 8): LPS + Ascorbic acid + L-histidine in dairy cattle. In D0 and D1, difference between OO and OH approaches statistical significance ($P < 0.10$).

APPENDIX D

ASCORBIC ACID PROTOCOL: ELECTROCHEMICAL DETECTION/HPLC ANALYSIS OF ASCORBIC ACID

Animal Health Diagnostic Laboratory, Nutrition Section, Michigan State University

Materials:

Ascorbic acid (Sigma A-7506), dodecyltrimethylammonium bromide (Sigma D-8638), ethylenediaminetetraacetic acid disodium salt (EDTA, Baker), methanol (Baxter), o-phosphoric acid (85%, Fisher), sodium acetate (Baker 3470-01), Sodium phosphate (monobasic anhydrous, and Sigma S-0751) were used without further purification.

Specifications

1) Ascorbic Acid ($C_6H_8O_6$) MW: 176.12

2,3-endiol-L-gulonic acid-gamma-lactone (Ascorbic acid)

$pK_1 = 4.17$ $pK_2 = 11.57$

Both free acid and salt are colorless, crystalline, highly water-soluble; not stable at pH > 10. Absorbance UV_{max}: 245 nm at acid medium; 265 at neutral medium.

HPLC system:

The HPLC system developed to measure ascorbic acid uses isocratic mobile phase buffer delivering and reverse phase C18 column coupled with electrochemical detection.

Mobile Phase:0.05 M sodium phosphate,

0.05 M sodium acetate,

300 mg/l dodecyltrimethylammonium bromide

40 μM EDTA

in 5% methanol in water (v/v), pH 4.8.

Column: 3.9 X 150 mm Nova-Pak C18 60 A 4 μm (Waters) and a guard-pak cartridge Holder with Nova-Pak C_{18} guard-pak precolumn insert (Waters)

Detector: ESA Coulochem II Multi-electrode Detector, Model 5200

Analytical Cell: ESA Model 5010

Guard Cell: ESA Model 5020

Approximate Settings:

	Potential	Current	
Guard cell:	350 mV		
Electrode 1:	-200 mV	≥-400	1 to 5 μA depending on samples
Electrode 2:	300 mV	≤300	1 to 5 μA depending on samples

Dummy cell is not connected

HPLC Mobile Phase Preparation

Mobile Phase Solution:

0.05 M sodium phosphate (NaH_2PO_4)

0.05 M sodium acetate (CH_3COONa)

300 mg/l dodecyltrimethylammonium Br

40 μM EDTA (disodium salt)

5% (v/v) methanol in H_2O; pH: 4.8

For 2 Liter Solution:

1) Add 1800 ml of fresh double distilled water in a 2 L beaker.

2) Add and stir: 13.8 g $NaH_2PO_4 \times H_2O$ (12.0 anhydrous)

8.2 g CH_3COONa (anhydrous)

600 - 800 mg dodecyltrimethylammonium Br

3) Add 29.8 mg disodium EDTA salt and stir (Do not use a higher concentration of EDTA as it will increase background current)

4) When EDTA is totally dissolved, add 100 ml of 100% methanol.

5) Transfer solution to a 2000 ml graduated cylinder and q.s. to 2000 ml with double distilled water.

6) Adjust pH to 4.8 with 85% o-phosphoric acid (about 3 ml)

7) Filter with 0.2 μm Nylon-66 filter (Rainin, vacuum filtration) into a 2 liter vacuum flask. Leave the vacuum on for another 20 min for degassing.

HPLC Standards

Standard Buffer: 0.05 M sodium phosphate (NaH_2PO_4)

0.05 M sodium acetate (CH_3COONa)

0.1 mM EDTA (disodium salt)

5% (v/v) methanol in H_2O; pH: 4.8

For 500 ml solution:

1) Add 400 ml double distilled water to a beaker.

2) Add and stir: 3.45 g $NaH_2PO_4 \times H_2O$ (3.0 anhydrous)

2.05 g CH_3COONa

3) Add 18.6 mg EDTA disodium salt and stir.

4) Add 25 ml of 100% methanol.

5) q.s. to 500 ml in a 500 ml graduated cylinder with double distilled water.

6) Adjust pH to 4.8 with 85% o-phosphoric acid (about 0.75 ml)

7) Filter with 0.2 μm Nylon-66 filter (Rainin, vacuum filtration) and degas with vacuum for an additional 20 min.

8) Seal and store at 4 °C.

Standard Solutions (keep tubes in ice):

1) Stock solution A

a) Weigh out 5 mg ascorbic acid into foil wrapped test tube.

b) Add 10 ml standard buffer (always use newly-made cold standard buffer) This will leave a 500 μg/ml solution.

2) Stock solution B

a) Take 40 μl of stock solution A and put into a foil wrapped test tube.

b) Add 9.96 ml standard buffer, and this will leave a 2 μg/ml (or 20 ng/10 μl) solution.

3) Stock solution C

a) Weigh out 1 mg uric acid and put into a foil wrapped 50 ml volumetric flask.

b) Add 50 ml standard buffer (always use newly-made cold standard buffer) and mix well. This will leave a 20 μg/ml (or 200 ng/10 μl) solution.

Note: the solubility of uric acid in water is very limited. Do not try to make a higher concentration.

4) Stock solution D

a) Take 1 ml of stock solution C and put into a foil wrapped test tube.

b) Add 9 ml standard buffer. This will leave a 2.0 µg/ml (20 ng/10 µl) solution.

5) 2 mix standard solution (AAUA-1010)

Mix equal volumes of stock solution B and D (10 ng ascorbic acid and 10 ng uric acid per 10 µl (AAUA-1010)) Place sample in micro tubes.

6) AAUA-1010 is stored at -80 °C and is good for at least a month.

Sample Preparation

Tissue Buffer:

90% methanol in water saturated with EDTA (final concentration is about 1 mM) Add 37.2 mg EDTA in 100 ml of 90% methanol/water solution, stir for 20 min and store in refrigerator.

Plasma Preparation

1) Bleed animals and collect blood in vacuum tubes which contain heparin (an alternative is to collect blood into EDTA tubes, but heparin gives better results)

2) Centrifuge in a table-top microfuge at 3000 rpm at 4 °C for 15 min.

3) Label 1.5 ml plastic microcentrifuge tubes.

3) Transfer 200 µl of supernatant (plasma) to each microcentrifuge tube.

4) Store in ultra-low freezer (-80 °C) or analyze for ascorbic acid as described in the following sections.

Measurement of Ascorbic Acid

Precipitation:

1) Remove 6-8 samples from ultra-low freezer and place in a rack in ice-cold water to

thaw the plasma or continue from the previous section.

2) One volume of plasma is mixed with two volumes (400 µl in this case) of tissue buffer.

3) Vortex and incubate on ice for 10 min.

4) Centrifuge at 3000 rpm, 4 °C for 15 min.

5) Transfer supernatant to another set of plastic microtubes and place them on ice (ascorbic acid are stable only for a couple of hours on ice, so do not prepare too many tubes each time)

Analyzing with HPLC-EC

1) Ready the column and analytical cell for the mobile phase by:

   a. Running in filtered 50% MeOH-water for 30 min at .5 ml/min.

   b. Running in fresh filtered 5% MeOH/distilled water 30 min at 1.0 ml/min.

   c. Running fresh filtered mobile phase to equilibrate overnight at 1.0 ml/min. Take waste tube from MeOH waste container and place it into the mobile phase container to recycle. Do this only when you are sure the mobile phase has passed through the entire system.

2) Daily, prior to running any samples, rinse pump seal with 10% MeOH.

   It is important to mix samples prior to each injection.

3) Inject 10 µl AAUA1010 standard before starting injection of samples; run two or more standard injections.

4) Inject 10 µl prepared samples onto HPLC column. Include Canine Plasma 1 if possible.

5) At the end of sample injections or the end of the day, inject 10 µl AAUA1010 standard

two times. Leave equilibrating overnight if you will use it again the next day.

6) When finished with system:

a. reverse potentials on the electrodes for 10-15 min. while running mobile phase to waste.

b. run fresh filtered 5%MeOH/distilled water at 1ml/min for 30 min.

c. run filtered 50% MeOH/water at .5 ml/min for 30 min.

d. run filtered 100% MeOH at .5 ml/min for 1 hour.

e. run 50% MeOH for 30 min again and store in 50% MeOH/water.

Calculation of results (calculated automatically on Millenium)

1) Place results in a spreadsheet like Excel (Millenium) The internal and external standard value were used in the quantification and determined by the specific peak of each sample and AAUA, respectively to calculate the content of ascorbic acid in samples.

2) Content of ascorbic acid or uric acid divided by injection volume (10 μl) gives concentrations of these acids in the injected sample. Include dilution factor (3x -- due to addition of tissue buffer to precipitate protein)

3) Inter-assay coefficient of variation (%) was calculated by (standard deviation / mean of all samples) x 100. But intra-assay coefficient of variation (%) was based on any single sample at any single day for about 2-4 data basis and calculated in the same manner. In this study, inter- and intra-assay coefficient of variation are 13.6 % and 5.9 %, respectively.

4) Report units in mg/dl. They will be converted into mM concentrations on the report.

Troubleshooting

Due to electrode fouling, it is necessary to electrically set the condition of the electrodes periodically. The procedure is not outlined in the manuals. With mobile phase running to waste set electrodes to 1000 mV for 10-15 min. Then reverse the potential to -400 mV for 10-15 min. Finally, make a new HDVA after reconditioning.

APPENDIX E

PHYCOERYTHRIN FLUORESCENCE-BASED ASSAY FOR REACTIVE OXYGEN SPECIES

The assay for reactive oxygen species depends on the detection of chemical damage to phycoerythrin through the decrease in its fluorescence emission. The fluorescence of phycobiliproteins is highly sensitive to the conformation and chemical integrity of the protein and prosthetics. Under the appropriate conditions, in the presence of reactive oxygen species, the rate of loss of phycoerythrin fluorescence is an index of free radical damage. The effect of added compounds on the rate of this fluorescence loss is a measure of their ability to protect the protein (Glazer, 1990).

Reagents

Porphyridium cruentum B-phycoerythrin (B-PE; Sigma, St. Louis, MO), a very soluble protein (>10 mg/ml in the 75 m*M* sodium phosphate buffer, pH 7.0) used in this assay. This stock solutions can be stored in 4 °C for months. A 40 n*M* stock solution of the water-soluble free radical initiator 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH; MW 267; Polysciences) was prepared in the pH 7.0 buffer immediately before use and stored on ice. Contaminating metal ions were removed from 75 m*M* sodium phosphate buffer, pH 7.0, by passage through a 10-ml column of Chelex with 100 resin (50-100 dry mesh), sodium form (Sigma, St. Louis, MO).

Experimental procedures

The assay for measuring the AOA in plasma was previously performed (Glazer, 1988). The final reaction mixtures contained 0.85×10^{-8} *M* B-PE, 2 m*M* AAPH, and other additives in 75 m*M* phosphate buffer, pH 7.0 (made from 75 m*M* K_2HPO_4 and 75 m*M* NaH_2PO_4) at 37 °C in a final volume of 4 ml, in 12x75 mm round borosilicate glass tubes. The mixture was added to the control tube in the following order: 3.58 ml of phosphate buffer, 0.02 ml of 1.7×10^{-6} *M* B-phycoerythrin, and 0.4 ml of 40 m*M* AAPH. Into each sample tube, 0.2-ml diluted plasma was added in place of the same volume of buffer. All final dilution of plasma in all runs is 1:320.

The reaction was initiated by adding 0.4 ml of 40 mM AAPH (freshly prepared and stored on ice) to the other components in 3.60 ml at 37 °C. Compensation for the temperature drop due to this addition required approximately 2 min. The solution was excited at 525 nm using a #58 filter, and emission was read at 575 nm using a #23A filter. The emission intensity was adjusted to a readable range using the excitation window set at 10X. Fluorescence was measured at 37 °C in a Turner Model 112 Digital Fluorometer (Sequoia-Turner Corporation, Mountain View, CA) immediately before and at 5-min interval for 40 min after addition of AAPH. Sample antioxidant activity was converted to percentage inhibition using the formula given below:

% Inhibition = [(Change FL Control - Change FL Sample) / (Change FL Control)] × 100

APPENDIX F

SAS & OUTPUT

SAS PROGRAM EDITOR

```
*libname 'c:\ac';

*run;

Data one;

 set ac.commilk;

*define treatments;

If tx = 1 then AA=0;

if tx = 1 then hist=0;

if tx = 2 then AA=1;

if tx = 2 then hist=0;

if tx = 3 then AA=1;

if tx = 3 then hist=1;

if tx = 4 then AA=1;

if tx = 4 then hist=1;

*Define groups;

G3=(m12+h0+h12)/3;

G2=(h24+h36)/2;

G1=(h48+h60+h72+h84+h96)/5;

proc glm;

class period tx cow half;

model g1 g2 g3= tx cow half period;

repeated group 3 (1 2 3)/summary printe;
```

```
contrast 'AA' tx -1 1 -1 1;
contrast 'Hist' tx -1 -1 1 1;
contrast 'OO-OH' tx -1 0 1 0;
contrast 'OO-OC' tx -1 1 0 0;
contrast 'OO-CH' tx -1 0 0 1;
contrast 'CO-OH' tx 0 1 -1 0;
contrast 'CO-CH' tx 0 1 0 -1;
contrast 'OH-CH' tx 0 0 1 -1;
title 'AA & L-Histidine';
run;
```

SAS OUTPUT

AA & L-Histidine

General Linear Models Procedure

Class Level Information

Class	Levels	Values
PERIOD	4	1 2 3 4
TX	4	1 2 3 4
COW	8	2612 2813 2926 2937 2952 3049 3133 3268
HALF	2	1 2

Number of observations in data set = 32

General Linear Models Procedure

Dependent Variable: G1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	445.92015016	31.85143930	24.54	0.0001
Error	17	22.06521784	1.29795399		
Corrected Total	31	467.98536800			

R-Square	C.V.	Root MSE	G1 Mean

0.952851	10.22049	1.13927784	11.14700000	
Source	*DF*	*Type I SS*	*Mean Square*	*F Value*	*Pr > F*
TX	3	12.57249100	4.19083033	3.23	0.0486
COW	7	424.88535400	60.69790771	46.76	0.0001
HALF	1	0.00017904	0.00017904	0.00	0.9908
PERIOD	3	8.46212612	2.82070871	2.17	0.1287
Source	*DF*	*Type III SS*	*Mean Square*	*F Value*	*Pr > F*
TX	3	13.40584709	4.46861570	3.44	0.0403
COW	7	424.88535400	60.69790771	46.76	0.0001
HALF	1	0.83765716	0.83765716	0.65	0.4329
PERIOD	3	8.46212612	2.82070871	2.17	0.1287

Dependent Variable: G2

Source	*DF*	*Sum of Squares*	*Mean Square*	*F Value*	*Pr > F*
Model	14	245.83894152	17.55992439	6.78	0.0002
Error	17	44.05674598	2.59157329		
Corrected Total	31	289.89568750			

R-Square	C.V.	Root MSE	G2 Mean
0.848026	18.65801	1.60983642	8.62812500

Source	*DF*	*Type I SS*	*Mean Square*	*F Value*	*Pr > F*
TX	3	3.58623750	1.19541250	0.46	0.7130
COW	7	229.72368750	32.81766964	12.66	0.0001
HALF	1	1.42224516	1.42224516	0.55	0.4689
PERIOD	3	11.10677136	3.70225712	1.43	0.2692
Source	*DF*	*Type III SS*	*Mean Square*	*F Value*	*Pr > F*
TX	3	3.38019110	1.12673037	0.43	0.7309
COW	7	229.72368750	32.81766964	12.66	0.0001
HALF	1	0.68090402	0.68090402	0.26	0.6148

PERIOD	3	11.10677136	3.70225712	1.43	0.2692

Dependent Variable: G3

Source	*DF*	*Sum of Squares*	*Mean Square*	*F Value*	*Pr > F*
Model	14	423.12637277	30.22331234	12.91	0.0001
Error	17	39.81041577	2.34178916		
Corrected Total	31	462.93678854			

R-Square	C.V.	Root MSE	G3 Mean
0.914005	15.30817	1.53029055	9.99656250

Source	*DF*	*Type I SS*	*Mean Square*	*F Value*	*Pr > F*
TX	3	3.02667326	1.00889109	0.43	0.7336
COW	7	408.29011354	58.32715908	24.91	0.0001
HALF	1	11.41746810	11.41746810	4.88	0.0413
PERIOD	3	0.39211786	0.13070595	0.06	0.9821

Source	*DF*	*Type III SS*	*Mean Square*	*F Value*	*Pr > F*
TX	3	2.79063300	0.93021100	0.40	0.7567
COW	7	408.29011354	58.32715908	24.91	0.0001
HALF	1	4.32716270	4.32716270	1.85	0.1918
PERIOD	3	0.39211786	0.13070595	0.06	0.9821

Repeated Measures Analysis of Variance

Repeated Measures Level Information

Dependent Variable	G1	G2	G3
Level of GROUP	1	2	3

AA & L-Histidine

General Linear Models Procedure

Repeated Measures Analysis of Variance

Partial Correlation Coefficients from the Error SS&CP Matrix / Prob > |r|

DF = 17	G1	G2	G3
G1	1.000000	0.476577	0.463698
	0.0001	0.0455	0.0526
G2	0.476577	1.000000	0.535779
	0.0455	0.0001	0.0219
G3	0.463698	0.535779	1.000000
	0.0526	0.0219	0.0001

E = Error SS&CP Matrix

GROUP.N represents the contrast between the nth level of GROUP and the last

	GROUP.1	GROUP.2
GROUP.1	34.38924576	18.48803187
GROUP.2	18.48803187	38.99054271

Partial Correlation Coefficients from the Error SS&CP Matrix

of the Variables Defined by the Specified Transformation / Prob > |r|

DF = 17	GROUP.1	GROUP.2
GROUP.1	1.000000	0.504893
	0.0001	0.0326
GROUP.2	0.504893	1.000000
	0.0326	0.0001

Test for Sphericity: Mauchly's Criterion = 0.742153

Chisquare Approximation = 4.7711976 with 2 df Prob > Chisquare = 0.0920

Applied to Orthogonal Components:

Test for Sphericity: Mauchly's Criterion = 0.9947029

Chisquare Approximation = 0.0849795 with 2 df Prob > Chisquare = 0.9584

Manova Test Criteria and Exact F Statistics for the Hypothesis of no GROUP Effect

H = Type III SS&CP Matrix for GROUP E = Error SS&CP Matrix

S=1 M=0 N=7

Statistic	Value	F	Num DF	Den DF	Pr > F
Wilks' Lambda	0.15197217	44.6412	2	16	0.0001
Pillai's Trace	0.84802783	44.6412	2	16	0.0001
Hotelling-Lawley Trace	5.58015209	44.6412	2	16	0.0001
Roy's Greatest Root	5.58015209	44.6412	2	16	0.0001

Manova Test Criteria and F Approximations for the Hypothesis of no GROUP*TX Effect

H = Type III SS&CP Matrix for GROUP*TX E = Error SS&CP Matrix

S=2 M=0 N=7

Statistic	Value	F	Num DF	Den DF	Pr > F
Wilks' Lambda	0.69659119	1.0568	6	32	0.4084
Pillai's Trace	0.30915168	1.0361	6	34	0.4194
Hotelling-Lawley Trace	0.42731799	1.0683	6	30	0.4031
Roy's Greatest Root	0.40706511	2.3067	3	17	0.1132

NOTE: F Statistic for Roy's Greatest Root is an upper bound.

NOTE: F Statistic for Wilks' Lambda is exact.

Manova Test Criteria and F Approximations for the Hypothesis of no GROUP*COW Effect

H = Type III SS&CP Matrix for GROUP*COW E = Error SS&CP Matrix

S=2 M=2 N=7

Statistic	Value	F	Num DF	Den DF	Pr > F
Wilks' Lambda	0.31042105	1.8168	14	32	0.0800
Pillai's Trace	0.81363088	1.6656	14	34	0.1110

Hotelling-Lawley Trace	1.82180623	1.9519	14	30	0.0609
Roy's Greatest Root	1.56673825	3.8049	7	17	0.0115

NOTE: F Statistic for Roy's Greatest Root is an upper bound.

NOTE: F Statistic for Wilks' Lambda is exact.

Manova Test Criteria and Exact F Statistics for the Hypothesis of no GROUP*HALF Effect

H = Type III SS&CP Matrix for GROUP*HALF E = Error SS&CP Matrix

S=1 M=0 N=7

Statistic	Value	F	Num DF	Den DF	Pr > F
Wilks' Lambda	0.79035271	2.1221	2	16	0.1523
Pillai's Trace	0.20964729	2.1221	2	16	0.1523
Hotelling-Lawley Trace	0.26525789	2.1221	2	16	0.1523
Roy's Greatest Root	0.26525789	2.1221	2	16	0.1523

Manova Test Criteria and F Approximations for the Hypothesis of no GROUP*PERIOD Effect

H = Type III SS&CP Matrix for GROUP*PERIOD E = Error SS&CP Matrix

S=2 M=0 N=7

Statistic	Value	F	Num DF	Den DF	Pr > F
Wilks' Lambda	0.71389075	0.9789	6	32	0.4555
Pillai's Trace	0.30577154	1.0227	6	34	0.4274
Hotelling-Lawley Trace	0.37323212	0.9331	6	30	0.4859
Roy's Greatest Root	0.27195727	1.5411	3	17	0.2402

NOTE: F Statistic for Roy's Greatest Root is an upper bound.

NOTE: F Statistic for Wilks' Lambda is exact.

General Linear Models Procedure

Repeated Measures Analysis of Variance

Tests of Hypotheses for Between Subjects Effects

Source	DF	Type III SS	Mean Square	F Value	Pr > F	
TX	3	12.25154126	4.08384709	1.00	0.4162	
COW	7	1028.38350418	146.91192917	36.02		0.0001
HALF	1	1.32018762	1.32018762	0.32	0.5768	
PERIOD	3	13.08439260	4.36146420	1.07	0.3883	
Error	17	69.33787520	4.07869854			

Univariate Tests of Hypotheses for Within Subject Effects

Source: GROUP

					Adj Pr > F	
DF	Type III SS	Mean Square	F Value	Pr > F	G - G	H - F
2	101.76916158	50.88458079	47.28	0.0001	0.0001	0.0001

Source: GROUP*TX

					Adj Pr > F	
DF	Type III SS	Mean Square	F Value	Pr > F	G - G	H - F
6	7.32512993	1.22085499	1.13	0.3638	0.3639	0.3638

Source: GROUP*COW

					Adj Pr > F	
DF	Type III SS	Mean Square	F Value	Pr > F	G - G	H - F
14	34.51565086	2.46540363	2.29	0.0243	0.0246	0.0243

Source: GROUP*HALF

					Adj Pr > F	
DF	Type III SS	Mean Square	F Value	Pr > F	G - G	H - F
2	4.52553626	2.26276813	2.10	0.1378	0.1381	0.1378

Source: GROUP*PERIOD

Adj Pr > F

DF	Type III SS	Mean Square	F Value	Pr > F	G - G	H - F
6	6.87662274	1.14610379	1.06	0.4025	0.4024	0.4025

Source: Error(GROUP)

DF	Type III SS	Mean Square
34	36.59450439	1.07630895

Greenhouse-Geisser Epsilon = 0.9947

Huynh-Feldt Epsilon = 2.0540

Analysis of Variance of Contrast Variables

GROUP.N represents the contrast between the nth level of GROUP and the last

Contrast Variable: GROUP.1

Source	DF	Type III SS	Mean Square	F Value	Pr > F
MEAN	1	42.35220613	42.35220613	20.94	0.0003
TX	3	11.33778106	3.77926035	1.87	0.1733
COW	7	9.17931421	1.31133060	0.65	0.7112
HALF	1	8.97253772	8.97253772	4.44	0.0504
PERIOD	3	7.63272646	2.54424215	1.26	0.3203
Error	17	34.38924576	2.02289681		

Contrast Variable: GROUP.2

Source	DF	Type III SS	Mean Square	F Value	Pr > F
MEAN	1	59.92387812	59.92387812	26.13	0.0001
TX	3	0.85442009	0.28480670	0.12	0.9445
COW	7	43.60529687	6.22932812	2.72	0.0438
HALF	1	1.57506076	1.57506076	0.69	0.4188
PERIOD	3	9.24101001	3.08033667	1.34	0.2936

Error	17	38.99054271	2.29356134

General Linear Models Procedure

Dependent Variable: G1

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
AA	**1**	**10.39184007**	**10.39184007**	**8.01**	**0.0116**
Hist	**1**	**1.02102050**	**1.02102050**	**0.79**	**0.3875**
OO-OH	1	3.21792600	3.21792600	2.48	0.1338
OO-OC	1	1.49940025	1.49940025	1.16	0.2975
OO-CH	1	2.53178904	2.53178904	1.95	0.1805
CO-OH	1	9.04547188	9.04547188	6.97	0.0172
CO-CH	1	0.14246730	0.14246730	0.11	0.7445
OH-CH	1	10.88542634	10.88542634	8.39	0.0100

Dependent Variable: G2

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
AA	**1**	**0.38623893**	**0.38623893**	**0.15**	**0.7042**
Hist	**1**	**2.96461250**	**2.96461250**	**1.14**	**0.2998**
OO-OH	1	1.20990007	1.20990007	0.47	0.5036
OO-OC	1	0.30802500	0.30802500	0.12	0.7345
OO-CH	1	0.58290358	0.58290358	0.22	0.6413
CO-OH	1	2.72271323	2.72271323	1.05	0.3197
CO-CH	1	1.72552200	1.72552200	0.67	0.4258
OH-CH	1	0.10755360	0.10755360	0.04	0.8410

Dependent Variable: G3

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
AA	**1**	**0.60724713**	**0.60724713**	**0.26**	**0.6171**
Hist	**1**	**1.37641701**	**1.37641701**	**0.59**	**0.4538**
OO-OH	1	0.04042907	0.04042907	0.02	0.8970

OO-OC	1	1.41015625	1.41015625	0.60	0.4484
OO-CH	1	0.07099087	0.07099087	0.03	0.8638
CO-OH	1	1.89917907	1.89917907	0.81	0.3804
CO-CH	1	2.08362975	2.08362975	0.89	0.3588
OH-CH	1	0.00405974	0.00405974	0.00	0.9673

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