The Effects of Ascorbic Acid on In Vitro Heterophil Function

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Abstract
As a feed additive, ascorbic acid has been shown to have a protective effect against bacterial and viral diseases and to reduce the impact of detrimental stress in chickens. This study examined the effect of ascorbic acid treatment on in vitro heterophil function by examining random migration and phagocytosis and bacterial killing of Staphylococcus au reus. Heterophils were evaluated in broiler chickens ranging from 5 to 16 wk of age, and age differences were seen. Significant increases in bacterial killing were found in heterophils treated with ascorbic acid, and this difference tended to be greater in chickens from 5 to 10.5 wk of age. No significant differences were found in phagocytosis or random migration, but ascorbic acid tended to decrease random migration. The most significant effect on in vitro heterophil function was an increase in bacterial killing.

Keywords
Veterinary Pathology, Veterinary Microbiology and Preventive Medicine

Disciplines
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The Effects of Ascorbic Acid on In Vitro Heterophil Function

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Received 11 December 1998

SUMMARY. As a feed additive, ascorbic acid has been shown to have a protective effect against bacterial and viral diseases and to reduce the impact of detrimental stress in chickens. This study examined the effect of ascorbic acid treatment on in vitro heterophil function by examining random migration and phagocytosis and bacterial killing of Staphylococcus aureus. Heterophils were evaluated in broiler chickens ranging from 5 to 16 wk of age, and age differences were seen. Significant increases in bacterial killing were found in heterophils treated with ascorbic acid, and this difference tended to be greater in chickens from 5 to 10.5 wk of age. No significant differences were found in phagocytosis or random migration, but ascorbic acid tended to decrease random migration. The most significant effect on in vitro heterophil function was an increase in bacterial killing.

RESUMEN. Efectos del ácido ascórbico sobre la función de los heterófilos in vitro.
El ácido ascórbico como aditivo del alimento ha demostrado tener un efecto protector contra enfermedades bacterianas y virales, lo mismo que para reducir el deterioro que causa el estrés en las aves. En este estudio se examinó el efecto del tratamiento con ácido ascórbico sobre la función (in vitro) de los heterófilos observando la migración al azar, la fagocitosis y la muerte bacteriana del Staphylococcus aureus. Los heterófilos fueron evaluados en aves de 5 a 16 semanas de edad. Se observaron diferencias por edad. Se encontraron diferencias significativas en la muerte bacteriana en los heterófilos tratados con ácido ascórbico y estas diferencias tuvieron la tendencia de ser mayores en las aves de 5 a 10.5 semanas de edad. No se observaron diferencias significativas en la fagocitosis o en la migración al azar pero se pudo observar la tendencia del ácido ascórbico de reducir la migración al azar. El efecto in vitro más significante sobre la función de los heterófilos fue el aumento de la muerte bacteriana.

Key words: ascorbic acid, bacterial killing, heterophil function, random migration, phagocytosis, Staphylococcus aureus

Abbreviations: CPM = counts per minute; EDTA = ethylenediaminetetraacetic acid; FBS = fetal bovine serum; HBSS = Hanks' buffered salt solution; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; OD = optical density; PBSS = phosphate-buffered salt solution; TSB = tryptic soy broth

Staphylococcal tenosynovitis and osteomyelitis are worldwide problems of broiler and broiler-breeder chickens and turkeys that result in increased condemnations at slaughter and decreased weight gain and egg production due to bacteremia and decreased mobility (20). This disease has resulted in economic losses in the poultry industry, and previous surveys have identified that staphylococcal tenosynovitis/arthritis and osteomyelitis account for a major proportion of musculoskeletal disease in poultry (28). The disease pathogenesis is poorly defined, but decreased leukocyte function, especially heterophils, may contribute to the chronic debilitating nature of the disease (5). This syndrome responds poorly to antibiotics and, so far, immunization has not been effective.

Critically important to both the human and veterinary medical communities is that staphylococcal bacteria are becoming resistant to antibiotics, e.g., the emergence of methicillin- and vancomycin-resistant strains of staphylococci.
An avian model has been used to examine avian heterophil function in staphylococcal tenosynovitis/arthritis/osteomyelitis (4,8). Acquired defects in avian heterophil function have been documented in association with both chronic staphylococcal infections and staphylococcal septicemia (5). Defects in neutrophil function have been documented in human beings with chronic bacterial infections including Staphylococcus aureus (2,18).

The use of ascorbic acid to improve cell function and protect against disease has been advocated in staphylococcal infections of humans (18,30) and various diseases of poultry (13,19). In humans, as chronic staphylococcal infections progress, plasma ascorbic acid concentrations decrease (18). We previously demonstrated that ascorbic acid concentrations decreased during experimental staphylococcal infections in poultry. In humans, ascorbic acid has been documented to upregulate neutrophil chemotaxis, phagocytosis, and bacterial killing as well as to result in clinical improvement in staphylococcal infections (18). As a means of therapy, ascorbic acid offers the important advantages of being nontoxic and economical and not conferring antibiotic resistance.

There is a need to determine if heterophil function, including phagocytosis and bacterial killing, can be increased by ascorbic acid treatment because this might contribute to the decreased mortality reported in the poultry literature. This study investigates the in vitro effects of ascorbic acid on heterophil random migration and phagocytosis and bacterial killing of S. aureus.

**MATERIALS AND METHODS**

**Preliminary data.** An in vivo pilot study was conducted to determine plasma ascorbic acid concentrations in 6-to-8-wk-old broiler chickens with experimental staphylococcal arthritis (1 × 10^6 S. aureus 921-1 intravenous inoculation) (4) without ascorbic acid feed supplementation (n = 4) and with ascorbic acid feed supplementation (n = 3). Controls consisted of healthy chickens fed diets without (n = 3) and with ascorbic acid supplementation (n = 4). The in vivo study compared chickens being fed a regular ration (Start and Growth; Purina Mills, Inc., St. Louis, MO) and a 1000 ppm ascorbic acid-supplemented (Hoffman-Roche, Inc., Nutley, NJ) ration with and without experimental staphylococcal infections. Plasma for ascorbic acid analysis was collected into ethylenediaminetetraacetic acid (EDTA) vacutainer tubes, placed on ice, and immediately centrifuged at 4 C; the plasma was collected and stored at −70 C until analyzed. Plasma ascorbic acid analysis was performed by reverse phase high-performance liquid chromatography with electrochemical detection at Michigan State University, College of Veterinary Medicine, Nutritional Laboratory, East Lansing, MI, and results were reported in micromoles per liter.

**Experimental design.** Heterophils were collected from clinically healthy nonvaccinated Arbor Acres × Ross broiler chickens with complete blood counts within normal reference intervals. Chickens were housed in the Iowa State University Laboratory Animal Resource Facility with 16 hr light and 8 hr dark cycles and provided growth ration and water ad libitum. Heterophils were harvested from chickens at ≤10.5 wk and ≥11.0 wk of age, and assays were repeated over a 12 wk time period. Paired samples were used in each assay, and repetitions for each assay type are indicated below. Heterophils were incubated with either ascorbic acid (1 × 10^{-1} M) or Hanks' buffered salt solution (HBSS) without calcium or magnesium with 0.1% fetal bovine serum (FBS). Assays for random migration, phagocytosis, and bacterial killing were conducted at 41 C.

**Heterophil separation and incubation.** Heterophils were collected from 5-to-16-wk-old chickens and separated by a slight modification of a previously described technique (3,7). Solutions and reagents used in the heterophil separation were prewarmed in an incubator at 41 C for 20 min. A volume of 6 ml of whole blood was collected into 12-ml syringes with a 1:9 ratio of 10% EDTA (ED 2SS; Sigma Chemical Co., St. Louis, MO) to blood. The blood was gently mixed with 4 ml of 1% methylcellulose (25 cps, M-6385; Sigma) in 15-ml polypropylene tubes, and then the sample was divided into two tubes with 5 ml each and centrifuged at 25 g. After centrifugation, the plasma, with theuffy coat layer above the erythrocytes, was removed into separate tubes, and the erythrocyte interface was gently washed with HBSS without calcium or magnesium (14175-020, Gibco-BRL, Grand Island, NY), with 0.1% fetal bovine serum (FBS) (Intergen Co., Purchase, NY), to harvest the remaining heterophils. The tubes were filled with HBSS with 0.1% FBS, mixed, and centrifuged at 375 × g for 10 min. After centrifugation, the supernatant was discarded and the cell pellets were resuspended in HBSS with 0.1% FBS to 3 ml and placed on Ficoll-Hypaque gradients (Sigma) (specific gravity 1.077, 3 ml; over specific gravity 1.119, 3 ml). After centrifugation for 25 min at 260 × g, the 1.077/1.119 interface and 1.119 band were retained, and the top layer, including the white band of mononuclear cells, was discarded. The tubes were filled with HBSS with 0.1% FBS, inverted, and centrifuged at 375 × g for 10 min. The cell pellets were resuspen-
ded to 3 ml in 1 50-ml polypropylene tube, then 25 ml of 0.87% NH₄Cl in 0.1% KHCO₃ was added, and the tubes were rocked for 7 min to lyse the remaining erythrocytes. After the 7 min, HBSS with 0.1% FBS was added for a total volume of 50 ml, mixed, and centrifuged for 15 min at 400 × g. The supernatant was discarded, and the cell pellet was resuspended to 2 ml and then divided into two 15-ml polypropylene tubes, filled with HBSS with 0.1% FBS, and centrifuged for 10 min at 375 × g. In the control tube, the heterophils were initially suspended to 1 ml in HBSS with 0.1% FBS and counted with an eosinophil unopette (Becton-Dickinson, Rutherford, NJ) and hemacytometer. Differential leukocyte counts were performed on cytospin preparations to exclude eosinophils from the final heterophil count, and then cells were suspended to an initial concentration of 3 × 10⁶ heterophils/ml. The heterophils in the ascorbic acid sample were suspended at the same cell concentration with 1 × 10⁻¹ M sodium ascorbate (A4034 l-ascorbic acid sodium; Sigma) in HBSS on the basis of previously published ascorbic acid concentrations (2) and incubated for 30 min at 21 C (2,31). After incubation, the cells were rinsed with HBSS with 0.1% FBS, centrifuged at 375 × g, and used for assays. The final heterophil concentration was adjusted for each assay. Heterophil purity (>95%) was examined by cytospin preparations and viability determined by trypan blue dye exclusion (>95%). In preliminary assays, the concentration of ascorbic acid was doubled, but no significant effect was noted (data not shown).

**Bacteria and pooled sera.** A characterized S. aureus strain 921-1, isolated from affected joints in a case of tenosynovitis, was used for heterophil/bacterial assays (6). Bacteria were kept frozen at −70 C and grown on blood agar plates; single colonies were harvested and grown in tryptic soy broth (TSB) overnight for 14 hr prior to each assay. For the bacterial killing assay, bacteria were washed three times for 15 min at 1290 × g at 4 C, opsonized for 20 min, and suspended to 5 × 10⁹ colony-forming units/ml in HBSS, standardized by spectrophotometry, and verified by colony counts. The ¹²⁵I labeling of S. aureus for phagocytic assays was performed as previously described (24) with slight modifications. Two milliliters of an overnight culture was inoculated into 500 ml of brain–heart infusion broth containing 500 µCi ¹²⁵I (Amersham, Arlington Heights, IL) and 10⁻³ M fluoroxyuridine (¹²⁵FUrD) (F0305; Sigma) and incubated for 24 hr at 37 C. After incubation, the bacteria were divided into 10 50-ml vials and centrifuged at 1000 × g for 20 min; the supernatant was removed and heat-killed for 1 hr at 56 C. Bacteria were then washed with phosphate-buffered salt solution (PBSS) 10 min at 450 × g, the supernatant was removed, bacterial counts were standardized by spectrophotometry, and ¹²⁵I-iododeoxyuridine–labeled bacteria were stored at −70 C until used.

Opsonization was done with pooled nonimmune avian sera collected from broiler chickens raised in Oregon and Iowa, filtered, and stored at −70 C. Serum titers against S. aureus were determined by microtiter plate agglutination, as previously described (3), to be 1:32.

**Random migration.** Random migration under agarose (n = 13 paired samples) was used to measure the ability of heterophils to move randomly (1,24). Agar plates were prepared with buffered sodium bicarbonate (NaHCO₃, 0.026 M, pH 7.2) added to 10 × M199 medium with Hanks’ salts and L-glutamine (11181-039; Gibco-BRL) containing 10% pooled normal chicken serum, 0.8% agarose (Indubiose A37 HAA; IBF Biotechnics Inc., Columbia, MD), and 1% penicillin-streptomycin solution (P0781; Sigma) (1). Five-milliliter amounts of the agarose medium were dispensed into 60 × 15-mm tissue culture petri plates (Becton-Dickinson Labware, Lincoln Park, NJ), allowed to solidify, and stored in an airtight container at 4 C with humidity. Heterophils were diluted to 5 × 10⁹ heterophils/ml and 10 µl was placed in pre punched 2.0-mm agar wells with samples run in duplicate. Plates were placed in a humidified incubator with 5% CO₂ at 41 C and incubated for 18 hr. After incubation, the plates were fixed with 8% glutaraldehyde for 60 min, the agar was removed, and adherent heterophils were stained with 0.1% crystal violet for 10 min, dried, and examined. The area of migration was determined by measuring the radius with a microscope ocular grid. The circular migration pattern (in millimeters) was calculated by the formula A = πR² and subtracting the area of the well from the total area.

**Phagocytosis.** The percentage of phagocytosis of S. aureus (n = 16 paired samples) was determined by measuring the ability of heterophils to ingest opsonized ¹²⁵I-iododeoxyuridine–labeled S. aureus (24). In preliminary studies, pooled normal chicken serum was compared with a previously characterized staphylococcal antisera (3) as an opsonin, and no significant differences were found in the phagocytic assay (data not shown). The S. aureus (100 µl), 50 µl of 1:10 dilution of pooled normal chicken serum, and Earle’s basic salt solution (24010-068; Gibco-BRL) were incubated for 15 min in a 41 C shaking water bath. Fifty microliters of a 2.5 × 10⁹ heterophil/ml suspension was added and shaken for 10 min. Then 0.5 ml of PBSS with 0.1 unit/ml of lysostaphin (L2898; Sigma) was added to the tubes and incubated for 30 min, after which 3.0 ml of cold PBSS was added to stop the reaction. Tubes were then centrifuged at 1250 × g at 4 C for 10 min and washed twice with PBSS. The supernatant was aspirated and discarded, and the final cell pellet, containing ingested labeled bacteria, was measured in a gamma coun-
ter to determine the counts per minute (CPM) of radioactivity. Standards included a standard tube with *S. aureus*, no heterophils, and no lysostaphin and a background tube with all reactants except heterophils. All assays were performed as duplicates.

**Bacterial killing.** Heterophils were collected from 21 paired samples. Assays were conducted at 41°C by the 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (MTT) method in 96-well microtiter plates (62409-068, Nunc plates; VWR Scientific Products, Batavia, IL) by slight modification of a published technique (7). All test and control wells were performed in quadruplicate. In this assay, the remaining live bacteria, after further cell incubation, reduce MTT and produce purple formazan. Bacteria were opsonized with 20% pooled chicken serum for 20 min. Standard curves were created with 100%, 50%, 25%, 12.5%, 6.25%, and 3.1% bacterial concentrations verified by plate colony counts. In separate wells, bacteria were incubated with 1 × 10^9 M sodium ascorbate (A4034 l-ascorbic acid sodium; Sigma) in HBSS to rule out direct killing of bacteria by ascorbic acid. Then 1 × 10^8 opsonized bacteria in 50 µl of HBSS were added to test and control wells. Test wells contained 3 × 10^7 heterophils in 100 µl HBSS with 0.2% FBS, and control wells contained the previously described contents without the bacteria. The plates were centrifuged (400 × g for 5 min) to increase bacteria and heterophil contact. Initially, incubation times of 0 (no further incubation after the 5-min contact time), 30, and 60 min were performed, and the 0- and 30-min incubations of heterophils and bacteria were optimal. These two incubation times were run for each assay. After incubation, plates were centrifuged at 600 × g for 10 min to pellet, and 120 µl of HBSS was removed from control and test wells. Sterile water (80 µl) was added to control and test wells to lyse the heterophils and incubated 5 min, and then 150 µl of TSB was added. A second incubation, found to be optimal at 2 hr in 5% CO₂ for bacterial amplification, was followed by the addition of 10 µl of MTT (M2128; Sigma) to each well, and incubated for 10 min to allow the color change due to formazan. Ten microliters of 0.04 N HCl in isopropanol was added to each well to dissolve the MTT (1:25 dilution) for 10 min. The plates were covered and stored overnight, and the optical density (OD) was read at 560 nm on an automated shaking microplate reader after sitting at 25°C for 30 min. Bacterial killing assay (MTT) results are reported as OD readings (7) with the lower OD reading indicating the fewer surviving bacteria.

**Statistical analysis.** The percentage of phagocytosis was converted to arcsines prior to statistical analysis (29). Values of OD readings and migration (in mm²) were used for the MTT and random migration analyses, respectively. For the heterophil function assays and plasma ascorbic acid concentrations, an analysis of variance was performed, and when significant differences among means were identified, Fisher's least significant difference was calculated for all paired means. Significant differences were examined in all age groups (5, 6, 6.5, 8, 10.5, 11, 13, 14, 15, and 16 wk of age), and statistical comparisons were made in chickens ≤10.5 wk and ≥11.0 wk of age. The alpha level was determined at 0.05 or 0.06, as indicated in results. For phagocytic assays, the mean percentages of phagocytosis ± SD were calculated to determine the percentages of phagocytosis with and without ascorbic acid treatment of heterophils.

Percentage of ingestion was determined as follows:

% ingestion = \[
\frac{1}{[(\text{CPM in reaction tube}) - (\text{CPM in background tube})]} + \frac{1}{[(\text{CPM in standard tube}) - (\text{CPM in background tube})]} \times 100.
\]

For the MTT assay, the percentage change in OD readings from incubation with ascorbic acid was calculated as

% change in OD = \[
\frac{1}{[(\text{OD without ascorbic acid treatment}) - (\text{OD with ascorbic acid treatment})]} + \frac{1}{\text{OD without ascorbic acid treatment}} \times 100.
\]

Also for the MTT assay, the percentage of bacterial killing was calculated as

% bacterial killing = \[
\frac{1}{[(\text{OD bacterial control}) - (\text{OD test - OD cell control})]} + \frac{1}{\text{OD bacterial control}} \times 100.
\]

**RESULTS**

**Preliminary data.** The plasma ascorbic acid concentrations were increased in both healthy chickens and staphylococcal-infected chickens when supplemented with ascorbic acid, as opposed to the same groups without supplementation. The means ± SD for plasma ascorbic acid concentrations were as follows: healthy control chickens with ascorbic acid supplementation = 133.99 ± 23.17 μM/liter; healthy control chickens with no ascorbic acid supplementation = 68.51 ± 13.16 μM/liter; staphylococcal-infected chickens with ascorbic acid supplementation = 81.95 ± 18.17 μM/liter;...
liter; and staphylococcal infection with no ascorbic acid supplementation = 23.28 ± 4.70 μM/liter (Fig. 1). Means were significantly different (P ≤ 0.05) when all groups were compared with the exception of the comparison of healthy control chickens compared with staphylococcal-infected chickens with ascorbic acid supplementation.

**Heterophil separation.** Initially, not enough heterophils were collected on the same day to run all assays from individual chickens, but a majority of chickens had all three assays performed. The collection of peripheral blood was increased to 12 to 24 ml to run multiple assays with no adverse effect on adult broilers. The heterophil separation procedure was followed with the addition of more tubes for the methylcellulose and gradient steps because it was important to maintain the stated blood to reagent ratios. The yield of heterophils from 6 ml of blood ranged from 4 × 10⁶/μl to 8.5 × 10⁷/μl, and from 12 ml of blood, the range was 8 × 10⁷/μl to 18 × 10⁷/μl. The critical factors for maximum heterophil yield were accurate centrifugation speeds and avoidance of any microclots in the blood samples.

**Random migration.** Over all age groups, no significant differences (P ≤ 0.05) were found in random migration between heterophils treated with ascorbic acid or HBSS, but comparisons of individual chickens (n = 4) did have significant differences (P ≤ 0.05). When age groups were examined, no significant differences (P ≤ 0.05) were noted. In all age groups, ascorbic acid tended to decrease random migration of heterophils (mean ± SD, 22.9 ± 12.7 mm²) compared with heterophils treated with HBSS (26.1 ± 15.6 mm²). Ten of 13 chickens assayed had decreased random migration when the heterophils were incubated with ascorbic acid.

**Phagocytosis.** For phagocytosis, no significant differences (P ≤ 0.05) were found between heterophils treated with ascorbic acid or HBSS for any age group. The means ± SD phagocytosis were 21.4 ± 7.7% for heterophils treated with HBSS and 20.4 ± 5.7% for heterophils incubated with ascorbic acid across all age groups.

**Bacterial killing.** In the MTT assay, a significant (P ≤ 0.05) increase in heterophil killing of S. aureus was seen when heterophils were incubated with ascorbic acid at 1 × 10⁻¹ M and compared with bacterial killing when incubated in HBSS. There was a trend (significant at P ≤ 0.06) for heterophils from chickens ≤10.5 wk of age to have greater increases in bacterial killing vs. chickens ≥11.0 wk of age. When heterophils were incubated with ascorbic acid, the mean percentage of decrease in OD for chickens ≤10.5 wk of age was 27.6% vs. 18.2% for chickens ≥11.0 wk of age. When ascorbic acid–incubated heterophils were compared with HBSS incubation, individual bird heterophils varied from 16% to 44% in the decrease of OD that corresponded to an increase in bacterial killing. For all age groups, bacterial killing was 64 ± 12% for heterophils treated with HBSS and 71 ± 12% for heterophils treated with ascorbic acid. No direct bacterial killing was observed when ascorbic acid was incubated with bacteria, but there was a slight trend for bacterial numbers to increase when compared with incubation in HBSS.
DISCUSSION

The use of ascorbic acid to improve cell function and protect against disease has been advocated in both human medicine (18,23) and veterinary medicine, including poultry medicine (13,19). In poultry, decreased ascorbic acid concentrations have been associated with stress (16), and administration of ascorbic acid has been associated with a protective effect against stress, e.g., corticosterone release (13,27). In poultry, ascorbic acid added to feed or water has been shown to ameliorate the effects of Escherichia coli and infectious bronchitis virus (9,13,14). Recently, ascorbic acid was found to decrease mortality in broiler ascites and had no effect on performance parameters (17).

A trend for ascorbic acid to decrease random migration of heterophils was found in our study. The effect of ascorbic acid may aid in keeping the inflammatory cells at the site of infection. In cattle, ascorbic acid decreased random migration (25). Additionally, studies in cattle demonstrated that the subcutaneous injection of ascorbic acid reversed the effects of dexamethasone alteration of neutrophil migration, oxidative metabolism, and antibody-dependent cytotoxicity (25).

We did not see differences in the percentage of phagocytosis in heterophils treated with ascorbic acid and those treated with HBSS. The percentage of phagocytosis was similar, but slightly decreased, compared with previous reports of staphylococcal ingestion by avian heterophils (3,4,7). These differences could be due to the assay type because prior assays used flow cytometric evaluation of phagocytosis (3,7). In cattle, ascorbic acid tended to enhance S. aureus ingestion by bovine neutrophils (25). Follow-up studies were not performed to examine the use of ascorbic acid in disease prevention or as an additive to cattle feed. What effect the rumen microflora might have on ascorbic acid metabolism is unknown.

Of the assays used, significant differences were seen in cell-associated bacterial killing with the MTT assay. Even though the cells are washed in the MTT assay, complete removal of external bacteria cannot be guaranteed, and hence the assay is categorized as cell-associated bacterial killing. Because subsequent bacterial amplification is performed, the changes in OD readings are probably a more accurate reflection of heterophil-associated bacterial killing than comparisons of percentage of bacterial killing. Because ascorbic acid apparently will increase \textit{in vitro} staphylococcal killing, the mechanism of ascorbic acid may be directly intracellular, extracellular, or both. The importance of ascorbic acid function as an extracellular antioxidant protecting the granulocyte, and possibly prolonging its functional life span, may be more critical for neutrophils that contain myeloperoxidase than for heterophils that primarily kill organisms via oxygen-independent pathways (11).

We found decreased plasma ascorbic acid concentrations in chickens with staphylococcal arthritis/tenosynovitis (Fig. 1). As chronic infections progress, plasma ascorbic acid levels decrease, possibly because of increased utilization (18,26). Between 150 and 1000 ppm of ascorbic acid in poultry feeds have been shown to decrease mortality under different disease and stress conditions (13,16,19). Recently, more stable formulations for ascorbic acid are available, and recommendations for supplementation amounts have changed (Dr. Bond, Roche, Ames, IA, pers. comm.). Generally, ascorbic acid is not regarded as a dietary requirement for poultry because poultry can synthesize the vitamin to meet body requirements under normal conditions (15,27). The natural ascorbic acid content of grains and feedstuffs is often below 10 ppm, but increased plasma ascorbic acid levels are rapidly achieved with feed or water supplementation within 8 hr (22,26). From prior studies, it appears that the longer the supplementation is given, the longer the plasma ascorbic acid concentrations may be sustained.

The importance of ascorbic acid for granulocyte function has been documented in humans. Neutrophils from humans have been shown to accumulate significant amounts of ascorbic acid intracellularly (10,31). When bacterial infections occur, granulocytes go to the site of infection, and their functional capability appears to be related to ascorbic acid concentration. Depletion of ascorbic acid results in a reduction of phagocytosis and killing (26). Theories for ascorbic acid upregulation of neutrophils have included prevention of autooxidation from neutrophil granules at the site of infection and improvement of phagocytosis and chemotaxis by modulation of tubulin tyrosi-
nolation (18); however, the mechanism remains unknown.

Defects in neutrophil function have been documented in humans with chronic bacterial infections including S. aureus (2,12,18,21), and decreased heterophil chemotaxis was found in a field case of chickens with S. aureus (6). In human patients with S. aureus infections, supplementation with ascorbic acid resulted in upregulation of neutrophil function, including chemotaxis, phagocytosis, and bacterial killing, and clinical improvement (2,18,23,30). In patients with neutrophil function defects and concurrent bacterial infections, in vitro incubation of neutrophils with $1 \times 10^{-1}$ M sodium ascorbate restored neutrophil function motility (2). In our studies of poultry, heterophil function was evaluated in clinically healthy chickens, and the effect of ascorbic acid may not be as important in upregulation of cell function in healthy animals vs. animals with chronic disease that may have decreased concentrations of ascorbic acid.

For these reasons, the literature tends to support that poultry feed supplementation with ascorbic acid is beneficial, especially in the face of stress or disease, but the mechanisms, other than general assumptions about antioxidant effects, remain undefined (19). Because cortisol-type hormones are known to suppress mammalian neutrophil (12) and heterophil function (13), the beneficial effects of ascorbic acid during stress, such as decreased mortality, may be due to upregulation of heterophil function and protection against infectious diseases. Our studies indicate that in vitro bacterial killing by heterophils is upregulated by ascorbic acid; therefore, prevention or amelioration of poultry staphylococcal infections with ascorbic acid supplementation may be possible. This would indicate a need to evaluate the effects of ascorbic acid on heterophil function during a disease such as S. aureus; however, obtaining data from these types of experimental studies can be complicated by the fact that heterophil function can be upregulated by the short-term infectious process (4). Because of public concern about antibiotic use in meat products and because antibiotic-resistant strains of bacteria are increasing, ascorbic acid is an intriguing candidate to serve as a natural, acceptable feed additive that may aid in disease prevention.

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Ascorbic acid effects on heterophil function


ACKNOWLEDGMENTS

This study was funded by USDA Formula Funds administered by Iowa State University. We thank Allison Florance for statistical analysis. Preliminary feeding trial data were funded by the Oregon State College of Veterinary Medicine, and we acknowledge Anita Sonn for technical assistance in that project.