Original Article

Effect of prophylactic administration of vitamin C in chickens with staphylococcal septic arthritis

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Abstract

Background: Septic arthritis (SA) due to Staphylococcus aureus is a major cause of lameness in poultry with improper response to antimicrobial therapy.

Objectives: The study evaluates the effect of prophylactic administration of vitamin C on SA induced by methicillin resistant S. aureus in chickens.

Methods: One hundred and twenty chickens were randomly assigned into four groups: I. Negative control (NC), II. Positive control (PC) with SA induced at the age of 35 days by intra articular injection of S. aureus. III. Vehicle control (VC) and IV. Arthritic vitamin C-treated (VitC) group (15 g/100 L of drinking water from day 25 to the end of the experiment). Samplings were performed on day 44 (sampling 1) and day 54 (sampling 2) of age.

Results: Arthritic birds showed an obvious decrease in body weight with severe clinical arthritis and lameness which were not significantly affected by vitamin C administration at both samplings. Moreover, marked increase in serum malondialdehyde (MDA) concentration of the PC group was observed in sampling 1. Administration of vitamin C successfully reduced MDA concentration at both samplings. In sampling 2, birds in the VitC group showed significantly higher total antioxidant capacity (TAC) than NC birds (p < 0.05). Interleukin-6 concentration in synovial fluid of chickens remained statistically similar among groups in both samplings, while histopathological changes were ameliorated in the VitC group in sampling 2.

Conclusions: Prophylactic administration of vitamin C especially for relatively longer period can ameliorate oxidative stress and histopathological changes due to staphylococcal arthritis in chickens, although it is not associated with a significant effect on clinical manifestations of the disease.

KEYWORDS
chicken, oxidative stress, S. aureus, septic arthritis, vitamin C
1 | INTRODUCTION

Staphylococcus aureus is a major cause of septic arthritis (SA) in veterinary and human patients. In humans, the disease has a debilitating and rapidly progressive nature. The prognosis is poor even in cases with proper antimicrobial therapy, and permanent joint destruction is common (Majd et al., 2020).

Chickens are a good model for S. aureus-induced SA and S. aureus is associated with bacterial chondronecrosis with osteomyelitis followed by lameness in broilers, which mostly occurs around 35 days of age. When established, the disease shows improper response to antibacterial therapy which reflects the importance of preventive measures (McNamee & Smyth, 2000; Wideman et al., 2015). Since 1990, Daum et al. (1990) described a chicken model of S. aureus SA and osteomyelitis, and different studies have used this avian species for the induction of S. aureus SA by different routes, including direct injection of bacteria into the joint (Gu et al., 2013; Mosleh et al., 2016; Zhou et al., 2017).

A fast recruitment of immune cells such as polymorphonuclear granulocytes and activated macrophages is observed in the infected joint followed by the T cells and the production of an array of cytokines. Macrophage-derived cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 have a prominent role in the evolution of severe inflammation that can lead to cartilage and bone destruction (Colavite & Sartori, 2014).

The inflammation can also promote oxidative stress. It is well established that oxidative stress and reactive oxygen species (ROS) have a pivotal role in osteoarthritis progression. The main source of oxidative stress in osteoarthritis is the peroxidation of chondrocytes-derived lipids. This leads to the generation of reactive products such as malondialdehyde (MDA) that attack cellular components and extracellular matrix with resultant adverse changes in cellular function and membrane permeability and also matrix protein degradation (Lepetos & Papavassiliou, 2016). Therefore, antioxidants have attracted interests in order to fight against osteoarthritis. Vitamin C or ascorbic acid is a well-known antioxidant. This vitamin is also important in bone and cartilage development and health by different mechanisms including a change in gene expression in bone cells (Aghajanian et al., 2015). Vitamin C has shown strong immunomodulatory effects that involve both innate and acquired immune responses (Mousavi et al., 2019). Moreover, growth-inhibiting effect of this vitamin against bacterial agents including methicillin-resistant S. aureus (MRSA) is reported (Mirani et al., 2018).

Although it may seem that this vitamin can have a beneficial effect in arthritis, its effects on different types of the disease show some inconsistency.

More than 70 years ago, in a pioneer study, Brownlee (1950) observed a slight protective effect of vitamin C in normal (but not in adrenalectomized) rats with formaldehyde-induced arthritis. Later, the protective effects of vitamin C against the development of arthritis in adjuvant-injected rats was described by Sakai et al. (1999). In an interesting report by Mal et al. (2012) on mice with experimental S. aureus arthritis, the authors evaluated the effect of a single dose of gentamicin with or without vitamin C as well as vitamin C alone on different parameters of osteoarthritis. Co-administration of vitamin C and gentamicin was most efficient in reducing clinical manifestations of arthritis and amelioration of oxidative stress. Prophylactic vitamin C administration has also prevented osteoarthritis progress in a rat model of monosodium iodoacetate-induced osteoarthritis (Chiu et al., 2017). On the contrary, in a study by Kraus et al. (2004), long-term, high-dose ascorbic acid intake worsened spontaneous knee osteoarthritis in a guinea pig model although it previously had shown protective effects against surgically-induced osteoarthritis in this animal model (Schwartz et al., 1981). Consistently, Chaganti et al. (2014) reported that high circulating levels of vitamin C is associated with the increased incidence of whole knee radiographic osteoarthritis in human patients.

Taken together, the inconsistencies which were observed in the effect of vitamin C against different types of osteoarthritis, its versatile properties along with the persistent problem in the treatment of SA due to S. aureus in poultry, intrigued us to evaluate the effect of prophylactic chronic administration of this vitamin on different aspects of S. aureus SA experimentally induced in broiler chickens. Different criteria related to response of challenged chickens to therapy including clinical scores as well as serum oxidative stress parameters, concentration of IL-6 in synovial fluid and histopathological features were assayed in two sampling periods.

2 | MATERIALS AND METHODS

2.1 | Bacteria

The bacterium used in this study was a coagulase and catalase positive, beta haemolytic S. aureus isolated from bumble foot lesions of poultry. The genus and species of the bacterium was confirmed by polymerase chain reaction (PCR) to amplify a species-specific region of the DNA coding for rRNA by using the primer pair of (5′-3′: SAU GC CCA CAT TAG ACG AAT CA) and (5′-3′: SAU CGG GCA CCT ATT TTC TAT CT) as described previously by Riffon et al. (2001). The bacterium was methicillin resistant as detected by agar disk diffusion method as well as by PCR with primers mecA1 (GTA GAA ATG ACT GAA CGT CCG ATA A) and mecA2 (CCA ATT CCA CAT TGT TTC GGT CTA A) (Jonas et al., 1999).

To further characterize the bacteria, multilocus sequence typing (MLST) was performed by primers specific to seven housekeeping genes: carbamate kinase (arcC), shikimate dehydrogenase (araE), glycero kinase (glp), guanylate kinase (gmk), phosphate acetyltransferase (pta), triosephosphate isomerase (pip) and acetyl coenzyme A acetyltransferase (yqiL) according to the method described by Enright et al. (2000). The PCR conditions were primary denaturation (95°C, 4 min), secondary denaturation (95°C, 1 min), annealing (55°C, 1 min), primary extension (72°C, 1 min for 34 cycles) and final extension (72°C, 7 min). The amplified fragments were purified by ethanol precipitation and sequencing was performed by genetic analyzer 3500 (Applied Biosystems, USA). The bacterium was ST-5732 disseminated clonal complex 5 (CC5) methicillin-resistant S. aureus.
2.2 | Study design

One hundred and twenty 1-day-old Ross 308 chickens from both sexes were reared at the same conditions as indicated by Ross 308 broiler management manual. The birds had free access to commercial feed and tap water during the experiment. Birds were randomly assigned into four groups (n = 30 each) and treated as follows:

I. Negative control (NC) group: these birds received no treatment. II. Positive control (PC) group: arthritis was induced in this group at the age of 35 days by injecting 1 ml of a suspension of S. aureus bacteria containing $1.4 \times 10^8$ CFU in TSB medium in the right tibiotarsal joint. The proper bacterial concentration for injection was determined in a pilot study. III. Vehicle control (VC) group: 1 ml of sterile TSB medium was injected in the right tibiotarsal joint. IV. Arthritic vitamin C-treated (VitC) group: birds in this group received vitamin C (vitamin C 50%, Rooyan Darou, Iran) at the dosage of 15 g/100 L of drinking water (equivalent with 11.25 mg/kg body weight of vitamin C) as the routine dosage of vitamin C for chickens in stressful conditions suggested by the manufacturer, from day 25 of age which continued to the end of the experiment.

2.3 | Sampling and clinical scoring

On day 44 of age (sampling 1), birds were weighed and monitored for clinical signs. Lameness in birds was scored by defining 0: normal movement, 1: slight lameness, 2: difficulty in moving, 3: difficulty in moving and a tendency to sit, 4: severe lameness and 5: debilitating lameness with the bird remained in sitting position for most of the observation time. Severity of arthritis was also scored as described by Bremell et al. (1992) by macroscopic inspection with a score of 0–3 for the right tibiotarsal joint (0, normal; 1, mild swelling and/or erythema; 2, moderate swelling and erythema and 3, marked swelling and occasional ankylosis). Blood samples were collected from wing vein of 10 birds from each group for determination of oxidative stress parameters. Then, birds were euthanized by decapitation and samples from synovial fluid of the right tibiotarsal joint was collected for determination of IL-6 level. Moreover, the joint was fixed in 10% neutral buffered formalin for histopathological evaluation. The same procedure was performed on day 54 of age (sampling 2 of the remaining 20 birds of each group).

All procedures used in the study are approved by our institutional ethical committee and are in accordance with Directive 2010/63/EU.

2.4 | Determination of serum MDA concentration and total antioxidant capacity

Blood samples were coagulated at room temperature and sera were harvested after centrifugation at 3000 rpm for 10 min. Harvested sera were kept at $-40^\circ$C until use.

Serum MDA concentration and total antioxidant capacity (TAC) were determined by using kits prepared by Zell bio, Germany based on colorimetric assays as described by the manufacturer.

2.5 | Determination of IL-6 concentration in synovial fluid

Sandwich ELISA method was used to quantitatively assay IL-6 in chickens’ synovial fluids. The chicken IL-6 ELISA kit was prepared by Biossay Technology Laboratory, China with intra-assay and inter-assay coefficients of variation of <8% and <10%, respectively. The assay was performed according to the manufacturer’s instructions.

2.6 | Histopathological evaluation

Samples of affected joints were fixed in 10% neutral buffered formalin and decalcified in 5% nitric acid. After decalcification, samples were trimmed and routinely processed then embedded in paraffin. Sections of 5 $\mu$m from paraffin blocks were taken and stained with haematoxylin and eosin for examination under light microscope.

2.7 | Statistical analysis

All data were presented as mean ± SD for all groups. Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparison test with $p < 0.05$ as the level of significant difference. Data analysis and preparation of graphs were performed by Graph Pad Prism 6 software.

3 | RESULTS

3.1 | Body weight and clinical scorings

As demonstrated in Figure 1, induction of SA by S. aureus was associated with an obvious decrease in body weight of birds in the PC group as compared to the NC and VC groups in both sampling times ($p < 0.001$ for all cases). Administration of vitamin C resulted only in a trivial insignificant increase in body weight of birds compared to the PC group in both samplings ($p > 0.05$). Birds with SA in the PC and VitC groups showed severe lameness with scores of $4.5 \pm 0.83$ (mean ± SD) and $4.25 \pm 0.86$ in the first sampling and $4.6 \pm 0.51$ and $4.12 \pm 0.83$ at the second sampling, respectively. The scores were not significantly different between these groups at both sampling times ($p > 0.05$) (Figure 2).

The arthritis severity scores also showed no difference between the PC and VitC groups in both sampling times ($p > 0.05$), although the birds in both groups showed signs of partial recovery with lower score means in sampling 2 in both groups ($2.6 \pm 0.51$ in the PC
2.5 ± 0.54 in the VitC group at sampling 1 and 1.44 ± 0.52 in the PC group vs. 1.57 ± 0.53 in the VitC group at sampling 2) (Figure 3).

3.2 Oxidative stress parameters

As shown in Figure 4, induction of SA was associated with marked increase in serum MDA concentration of the PC group as compared to NC and VC birds in sampling 1 (p < 0.001, in all cases). Administration of vitamin C successfully reduced MDA concentration as compared to the PC group (p < 0.05), although it was not reversed to the NC group level (p < 0.01). In sampling 2, no significant difference was observed in serum MDA concentration among the NC, VC and PC groups (p > 0.05), while birds in the VitC group showed significantly lower serum MDA concentration as compared to these three groups (p < 0.01 for the PC group vs. the VitC group and p < 0.001 for the NC and VC groups vs. the VitC group).

Regarding TAC (Figure 5), both the PC and VitC groups showed increased TAC as compared to the NC and VC groups in sampling 1 (p < 0.001 for all comparisons). Birds in the VitC group had slightly increased TAC as compared to PC birds, which was not significant (p > 0.05). In sampling 2, TAC of the NC, PC and VC groups were statistically the same (p > 0.05). Birds in the VitC group showed significantly higher TAC as compared to NC birds (p < 0.05).

3.3 Level of IL-6 in synovial fluid

Figure 6 shows IL-6 levels of birds in different groups. No significant difference was observed in this parameter among groups in both samplings (p > 0.05).
3.4 Histopathological findings

In both samplings, birds in the NC and VC groups showed normal histological features of synovial membrane, articular cartilage and bone structures. Birds in the PC group demonstrated obvious septic fibrinoheterophilic synovitis or arthritis (Figure 7). Severe degeneration and necrosis of synovial membrane cells were present in the PC group at both samplings accompanied by hyperaemia, edema and fibrinoheterophilic exudate with intralesional bacterial colonies. Thickening of articular capsule due to edema, fibrin deposition and infiltration of heterophils especially around hyperaemic blood vessels was observed. Some samples showed erosions in surface of articular cartilage associated with infiltration of few heterophils. In sampling 1, birds in the VitC group showed severe changes including hyperaemia, edema, fibrin deposition and infiltration of heterophils similar to that of the PC group as well as severe degeneration and necrosis of synovial membrane. However, in sampling 2, the extent of degeneration and necrosis of synovial membrane was moderate in these birds, and the presence of heterophils and mononuclear cells was mostly restricted to perivascular areas (Figure 8). Moderate hyperaemia and slight edema were observed in this group on sampling 2, while fibrin deposition was vastly present.

4 DISCUSSION

In the present study, we investigated the effects of prophylactic administration of vitamin C against S. aureus experimental SA in broiler
FIGURE 5  Serum total antioxidant capacity (TAC) concentration (mean ± SD) of birds in different groups at the age of 44 (sampling 1) and 54 days (sampling 2). NC: negative control (no treatment); PC: positive control (birds with experimentally induced septic arthritis by injecting *S. aureus* in the tibiotarsal joint); VC: vehicle control (injected with bacterial culture medium in the tibiotarsal joint) and VitC: birds with experimental septic arthritis that were prophylactically treated with vitamin C. Columns with different letters have a significant difference at *p* < 0.05.

FIGURE 6  Synovial fluid IL-6 level (mean ± SD) of birds in different groups at the age of 44 (sampling 1) and 54 days (sampling 2). NC: negative control (no treatment); PC: positive control (birds with experimentally induced septic arthritis by injecting *S. aureus* in the tibiotarsal joint); VC: vehicle control (injected with bacterial culture medium in the tibiotarsal joint) and VitC: birds with experimental septic arthritis that were prophylactically treated with vitamin C. No significant difference was observed among the groups (*p* > 0.05).

Chickens. Lameness and arthritis severity scores were evaluated as clinical parameters for successful induction and possible suppression of SA by vitamin C in two samplings performed on days 10 and 20 post induction. As previously stated, induction of SA was associated with a marked increase in these scores and also a reduction in body weight at both sampling times that can be a reflection of reluctance of birds to move and reach feed.

In a related study by Mal et al. (2012), SA was induced in mice by intravascular injection of *S. aureus* in tail vein and swelling of wrist and ankle joints were evaluated. Consistently, a significant increase was observed in these parameters at days 3, 9 and 15 post induction. These researchers administered a single dose of vitamin C at the dosage of 20 mg/kg body weight by intra peritoneal injection 26 h after induction of arthritis. Although a significant change in wrist joint swelling was not observed in vitamin C-treated mice, the swelling of ankle joint was reduced due to vitamin C administration. Regarding our study, prophylactic vitamin C administration did not affect lameness and/or arthritis severity scores in birds with *S. aureus* SA. This relative discrepancy may originate from the difference in method of induction of SA in two studies (direct presentation of the bacteria to the joint which provides an ideal milieu for bacterial proliferation with resultant damages vs. haematogenous access of bacteria to joint) among other differences such as the species, the dose of vitamin C and dosing program.

As it was expected, induction of SA resulted in a drastic change in oxidative status parameters in sampling 1 with a prominent increase in serum MDA concentration of birds in the PC group, which was ameliorated by vitamin C administration in VitC birds. Production of ROS and oxidative stress are frequently reported in patients with osteoarthritis...
as well as animal models of the disease (Altay et al., 2015; Altindag et al., 2007; Ertürk et al., 2012; Henrotin et al., 2003; Stamler et al., 2001; Tiku et al., 1990). Oxidative stress has been implicated in the inhibition of new cartilage extracellular matrix synthesis as well as chondrocyte death. Function of osteoblasts and osteoclast are also affected by free radicals (Zahan et al., 2020).

Consistent with our results, in a study by Staurengo-Ferrari et al. (2017), where SA was induced by local injection of S. aureus into the right knee joints of mice, a significant increase in MDA concentration of knee joint was observed at days 7, 14, 21 and 28 post induction of arthritis. These authors also evaluated free radical scavenging (ABTS assay) and ferric reducing (FRAP assay) properties, which showed a decrease in arthritic mice as compared to the control group. In our study, we observed a significant increase in TAC of serum in groups with SA 10 days after induction, which can be a compensatory mechanism to resist oxidative damage. The positive effects of vitamin C administration on oxidative stress were prominent in sampling 2 with reduced MDA concentration along with increased TAC in birds of the VitC group. These positive effects on oxidative stress status were in line with ameliorated histopathological changes, which were observed in birds of the VitC group in sampling 2.

Vitamin C has a pivotal role at transcriptional and post-transcriptional levels for synthesis of collagen II as the major protein component of articular cartilage. In a study by McNulty et al. (2005) on human chondrocytes, the authors reported that chondrocytes are very adept in concentrating vitamin C in the reduced form and its metabolites at a level of 960-folds over the concentration in the extracellular milieu. The main source of oxidative stress in osteoarthritis is the peroxidation of chondrocytes-derived lipids (Lepetsos & Papavassiliou, 2016), therefore possible accumulation of high concentrations of vitamin C in chondrocytes after vitamin C administration may explain its beneficial effect on oxidative stress in birds with SA as observed in the present study.

Another parameter that was evaluated in our study was IL-6 concentration as a pro-inflammatory mediator in synovial fluid. The level of this cytokine did not show a significant difference among groups in both sampling times. In a study by Corrado et al. (2016), the authors evaluated the effect S. aureus SA induced by intravenous administration of the bacteria on pro-inflammatory cytokines level in knee joint washes of mice. They described a fast increase in IL-6 concentration with a peak on day 7 post infection, which returned to near baseline values on day 14 post infection. Therefore, the reason behind not detecting the IL-6 rise in PC birds in our study may at least partially be related to the sampling time. Interestingly, in a study by Mosleh et al. (2016), the authors also did not find a significant change in serum IL-6 levels of chickens 9 days after intra articular injection of S. aureus. Therefore, species differences may also be a determining factor in this regard.

A limitation of our study was the fact that we assessed the effect of a single dosage of vitamin C (15 g/100 L of drinking water), which is in the mid-range (12–20 g/100 L of drinking water) of the dosage usually administered in acute stressful conditions in chickens. Although chickens can efficiently synthesize vitamin C, their need to this vitamin increases considerably in stressor conditions (Mosleh et al., 2018). Consistently, the prevalence of vitamin C deficiency is high in human patients with severe conditions, such as surgical/trauma, infectious diseases and cancer. Interestingly, it has been shown that high doses of vitamin C has analgesic properties (Carr & McCall, 2017), which is a desired effect in patients with osteoarthritis for reducing clinical signs. Therefore, evaluation of the effects of other dosages of vitamin C especially on arthritis-associated pain may be quite intriguing.

In conclusion, prophylactic administration of vitamin C especially for relatively long period can ameliorate oxidative stress and histopathological changes due to induction of SA by S. aureus in chickens, although it is not associated with a significant effect on clinical manifestations of the disease.
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CONFLICT OF INTEREST
The authors declare no conflict of interest.

ETHICS STATEMENT
All procedures used in this study are in accordance with our institutional ethical guidelines for care and use of animals in experiments which are based on EU Directive 2010/63/EU for animal experiments.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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