

Gentamicin in Combination with Ascorbic Acid Regulates the severity of *Staphylococcus aureus* Infection—Induced Septic Arthritis in Mice

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Abstract

To study the effects of gentamicin in combination with ascorbic acid on septic arthritis, mice were infected with *Staphylococcus aureus* (*S. aureus*) and treated with gentamicin, which was given at 5 mg/kg after 24 h of infection, followed by ascorbic acid, given at 20 mg/kg body weight after 2 h of gentamicin treatment. Mice were sacrificed at 3, 9, 15 days post-infection (dpi). Combined treatment of infected mice with gentamicin and ascorbic acid eradicated the bacteria from the blood, spleen and synovial tissue and showed a significant gross reduction in arthritis, reduced serum levels of tumour necrosis factor alpha (TNF- α) and interferon gamma (IFN- γ). *S. aureus*-infected mice have demonstrated the disturbed antioxidant status measured in terms of cellular antioxidants like reduced glutathione and antioxidant enzymes such as superoxide dismutase (SOD) and catalase. The same were ameliorated when the animals were co-treated with gentamicin along with ascorbic acid.

Introduction

Intravenous inoculation of mice with an exotoxin (toxic shock syndrome toxin-1, that is, TSST-1) producing strain of *Staphylococcus aureus* leads to the development of synovial inflammation and systemic pro-inflammatory cytokines, subsequently inducing severe septic arthritis [1]. Synovial inflammation is a response of the organism to injury related to physical or chemical noxious stimuli or microbial toxins (TSST-1), which has been reported to be involved in multiple pathologies in septic arthritis [2]. Inflammatory processes during septic arthritis erode articular damage; destroy bone and cause degenerative changes in the joints leading to irreversible loss of joint functions [3]. *Staphylococcus aureus*-induced septic arthritis arises as a result of haematogenous seeding of joints or joint surgery, from a penetrating wound or from an adjacent site of osteomyelitis [3]. Joint inflammation can also be induced by bacterial products, immune complexes and crystals, which recruit and activate the phagocytes to primarily form reactive oxygen species (ROS) [4]. The inflammatory tissue injury may also result via an oxidant-induced cellular proliferation of pro-inflammatory cytokines, which may potentiate

further release of cellular oxidants [5]. The appearance of synovial inflammation characterized by an abundance of polymorphonuclear neutrophils and a very rapid destruction of bone and cartilage is also coherent with bacterial arthritis in human [6]. In case of staphylococcal arthritis, PCR demonstrated persistent *S. aureus* DNA in the synovial fluid for 10 weeks despite adequate antibiotic treatment and sterile synovial fluid [7]. Septic arthritis and sepsis are common and feared complications of staphylococcal infections and the increasing antibiotic resistance among staphylococci urge the extended research for virulence factors involved in these diseases [8]. Increased ROS have been documented at the sites of inflammation such as synovial joints of patients with inflammatory arthritis [9]. It has been reported that ROS destroy the antioxidant system in arthritic patients.

New strategies of combating septic arthritis as well as eradicating oxidative stress are necessary to evolve. Because the incidence of multidrug resistant *S. aureus* infection is increasing, it is clear that new strategies are needed to combat this insidious pathogen [10]. Early administration of antibiotics eradicates the bacteria but does not stop joint destruction [11]. Studies in mice using corticosteroids in

conjunction with antibiotics indicated that this approach did lead to decreased morbidity and mortality. It was also suggested that a combination of antibiotics with some biological agents may be a useful approach for the treatment of *S. aureus*-induced inflammation and consequential damage [12]. However, the literature concerning this approach of treatment is meagre and not currently practised by the clinicians despite its usefulness [13]. These therapies envision alternatives to direct microbial killing, such as blocking disease progression by neutralizing-specific virulence factors or boosting key innate immune defences [14]. A vast effort has been made to characterize the virulence factors of *S. aureus* with the aim of finding a more efficient treatment [15]. It was also suggested that new methods may be adopted to treat the ongoing infections with combinations of anti-inflammatory, anti-bone resorptive agents, passive immunization and antioxidants to minimize the risk of sequele [16].

Here, we hypothesize that ROS may modulate synovial inflammation by stimulating the synthesis of cytokines, which may be dependent on the type of ROS generated and the signalling pathways activated.

Ascorbic acid (vitamin C) has also been shown to protect rodent hepatocytes from lethal oxidative stress [17]. The effects of antibiotics in experimental infectious arthritis have been reported [18], and the systemic and local application of gentamicin in the animal models of osteomyelitis and peritonitis has also been reported [19, 20]. It has further been reported that if Gram staining does not show bacteria, yet septic arthritis is still suspected and the patients should be maintained on continued gentamicin therapy while waiting for the results of the culture [21]. Continuous infusion of gentamicin in the tarsocrural joint of horses for 5 days is an acceptable method for the treatment of septic arthritis [22, 23]. Gentamicin was also chosen as an antibiotic, as it has been successfully used as a locally applied antibiotic in orthopaedic surgery. Its broad antimicrobial spectrum covering most bacteria commonly involved in osteomyelitis, and its bactericidal effects make it favourable in our study. Therefore, identifying staphylococcal septic arthritis diseases whose profiles suggest an intracellular component and adapting new protocols like antibiotics plus antioxidants to combat intracellular *S. aureus* is clearly a priority. Herein, we report the results of our studies in relation to the efficacy of the treatment with gentamicin alone or in combination with ascorbic acid on the *S. aureus* infection-induced arthritis.

Materials and methods

Male Swiss albino mice, 6–8 weeks of age with body weight 20 ± 4 g, were purchased from regular animal suppliers to our department. Upon arrival, mice were randomized into plastic cages with filter bonnets and saw dust bedding, followed by a 1-week quarantine period. Six

mice were housed per cage with food and water ad libitum. Animal holding rooms were maintained at 21–24 °C and 40–60% humidity with a 12-h light dark cycle. The mice were fed with normal rodent diet.

Preparation of bacteria

Staphylococcus aureus AG-789 was obtained from Apollo Gleneagles Hospital, Calcutta, and was maintained in our laboratory and tested for antibiotic sensitivity. Bacteria were cultured on blood agar (5% human erythrocytes) for 24 h and then re-incubated on blood agar for another 24 h. Before experimentation, bacteria were grown overnight at 37 °C in 5 ml of Luria Bertini broth, were diluted in fresh broth and were cultured until mid-logarithmic phase of growth [24]. Bacteria were harvested, washed in sterile phosphate buffered saline (PBS) and adjusted to the desired inoculums [25] spectrophotometrically before infection ($OD_{620} = 0.2 = 5 \times 10^7$ cells/ml for *S. aureus*), and the colony forming unit (CFU) was confirmed by serial dilution and culture on blood agar.

Treatment of *S. aureus* (AG-789)-infected mice with antibiotic (Gentamicin) followed by vitamin C (ascorbic acid)

In a separate set of experiment, starting on day zero after injection of *S. aureus* (AG-789) (5×10^6 cells/ml), the antibiotic gentamicin was dissolved in sterile PBS and injected intraperitoneally into mice (comprises six mice per experimental group) at a single dose of 5 mg/kg after 24 h of infection [26]. The fresh solution of ascorbate was prepared on the day of antibiotic treatment, and 1 ml of ascorbate corresponding to 20 mg/kg body weight in mice was given intraperitoneally to the same mice after 2 h of antibiotic treatment [27]. Then, these mice were sacrificed at 3, 9 and 15 days post-infection.

Determination of number of viable *S. aureus* cells in blood and organs (spleen and synovial tissue)

Blood (0.5 ml) was obtained on days 3, 9 and 15 after *S. aureus* (AG-789) infection by retro-orbital sinus bleeding before sacrifice at selected intervals. The blood from each infected mice was plated on mannitol salt agar selective media. Then, the mice were sacrificed, and spleen tissues were aseptically removed and homogenized with 3 ml of sterile RPMI-1640. All wrist and ankle joints from each mouse were removed, weighed and homogenized in RPMI-1640 medium (1 ml per 100 mg joint weight). After homogenization, all tissue samples were diluted and plated in triplicate on mannitol agar, and the results were expressed as the number of CFU per whole organ or per ml joint homogenate. To avoid false-positive results due to contamination, an isolate was considered positive when 15 or more *S. aureus* colonies were present [28]. Bacteria

recovered from the blood or spleen were definitely *S. aureus*, because this culture media differentiates *S. aureus* from other catalase-positive and Gram-positive cocci like *S. epidermidis*. *S. aureus* are grown on agar medium containing 7.5% NaCl, where growths of other organisms are inhibited. *Staphylococcus aureus* also can ferment mannitol into acid detected and comprehended by the change in pH indicator from red to yellow. A number of bacterial CFU obtained from either blood or spleen were not false positive, because bacterial presence was defined as 15 CFU or more for blood or tissue that are even higher in our case.

Induction and assessment of septic arthritis

Briefly, AG-789, a TSST-1-positive *S. aureus* originally isolated from our previous models of murine septic arthritis, was stored in nutrient agar at 4 °C and before each experiment was cultured on 5% blood agar for 24 h at 37 °C. The cell suspension was standardized spectrophotometrically to contain 5×10^7 CFU/ml. Male mice aged 3–4 weeks received either 5×10^6 CFU *S. aureus* in 100 μ l PBS injected i.v. via the tail vein, or 100 μ l PBS alone. Individual mice were observed daily for up to 15 days, blind to infection status. Swelling of wrist and ankle joints was used to determine the level of the inflammatory response in mice challenged with *S. aureus*. Prior to experimentation, the paws of randomly selected and age-matched mice were measured to determine the baseline paw size. After infection, the mice were measured every other day for 15 days with a dial-type vernier calliper graduated 0.1 cm increments by carefully measuring the width and thickness of each wrist and ankle joints. The daily mean value for each the group by the number of wrists or ankles was measured in each group. This average value represented the severity of wrist and ankle joints swelling [29]. Criteria for determining morbidity/sickness in mice included hunched posture, decreased activity, ruffled fur and laboured breathing.

Percentage reduction in arthritis per group of treated animals was calculated as follows:

$$\left[\frac{\text{Mean diameter of swelling of the wrist or ankle on day 15} - \text{swelling of the wrist or ankle on day 15 after treatment}}{\text{Mean diameter of swelling of the wrist or ankle on day 15}} \times 100 \right]$$
 Differences in the reduction of joint swelling between groups of drug treated and untreated arthritic mice were evaluated statistically [30].

Sample preparation for cytokine measurement

Blood samples from mice infected with 5×10^6 cells/ml of *S. aureus* and from uninfected, untreated control mice were obtained by cardiac puncture under ether anaesthesia at selected intervals. The blood (0.2 ml) was transferred to micro-centrifuge tubes and allowed to clot at 4 °C. Blood samples were then centrifuged at $300 \times g$ for 5 min at

4 °C. The supernatant pale yellow coloured serum was pipetted out carefully with the help of micropipettes into fresh micro-centrifuge tubes, labelled and stored at –80 °C for cytokine analysis. In each experiment, the mice were coded to ensure that the observer was blinded. Serum from different groups were normalized to the protein content by Bradford method before the assay and levels of cytokines (IL-6, IL-10, TNF- α and IFN- γ) were determined by Sandwich ELISA according to the manufacturer's instruction in a BioRad ELISA Reader.

Articular neutrophil accumulation

Myeloperoxidase (MPO) enzyme activity was analysed as an index of neutrophil infiltration in the synovial tissue, because it is closely related to the number of neutrophil present in the tissue. Synovial tissues were separated from mouse limb-joints and were first homogenized in 10 volumes of a buffer containing 20 mM Tris-HCl, (pH 7.0), EDTA, sucrose and protease inhibitor cocktail and then centrifuged at $2,000 \times g$ for 10 min at 4 °C. The supernatants were sterilized by passing through a Millipore filter (0.45 μ m pore size) and stored at –80 °C until analysis. Protein levels in the tissue homogenates were determined by Lowry method. An aliquot of the supernatant was allowed to react with a solution of O-dianisidine dihydrochloride (0.167 mg/ml) and 0.005% H₂O₂. The rate of change in absorbance was measured spectrophotometrically at 405 nm. MPO enzyme activity has been defined as the concentration of enzyme degrading 1 μ M of peroxide/min at 37 °C and was expressed as change in absorbance/min/mg of protein [31].

Estimation of tissue protein

Protein content of tissue homogenates supernatant and serum was estimated by Folin reaction according to Lowry *et al.* 1951 [32].

Measurement of lipid peroxidation level (LPO)

The weighed amounts of the hepatic, heart, kidney and spleen tissues were homogenized (10%) in ice-cold 0.9% saline (pH 7.0) with a Potter Elvehjem all glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30 s, and the levels of the lipid peroxidation products in the homogenate were determined as Thio-Barbituric Acid Reactive Substances (TBARS). In brief, the homogenates were mixed with trichloro acetic acid-thiobarbituric acid-hydrochloric acid (TBA-TCA-HCl) reagent and mixed thoroughly and heated for 20 min at 80 °C. The tubes containing the samples were then cooled to room temperature. The absorbance of the pink chromogen present in the clear supernatant after centrifugation at $1200 g$ for 10 min at room temperature was measured at 532 nm using a UV-VIS spectrophotometer.

(SmartSpec Plus, BioRad, Hercules, CA, USA). Tetraethoxypropane was used as standard. The values were expressed as nmoles of TBARS per mg protein [33].

Measurement of reduced glutathione level (GSH)

Reduced glutathione content (as acid soluble sulfhydryl) was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlac and Lindsey with some modifications. The weighed amounts of the hepatic, heart, kidney and spleen tissues were homogenized (10%) in 2 mM ice-cold ethylenediaminetetraacetic acid (EDTA). The homogenates were mixed with Tris-HCl buffer, pH 9.0, followed by the addition of DTNB for colour development. The absorbance was measured at 412 nm using a UV-VIS spectrophotometer to determine GSH content. The values were expressed as nmoles of GSH per mg protein [34].

Measurement of activity of antioxidant enzymes

Superoxide dismutase (SOD)

Superoxide dismutase (Cu-Zn SOD) activity was measured by haematoxylin auto oxidation method of Martin *et al.* In brief, the weighed amounts of the hepatic, heart, kidney and spleen tissues were homogenized (10%) in ice-cold 50 mM phosphate buffer containing 0.1 mM EDTA pH 7.4. The homogenates were then centrifuged at 12000 × g for 15 min, and the supernatant was carefully collected. The inhibition of haematoxylin auto oxidation by the cell-free supernatant was measured at 560 nm using a UV-VIS spectrophotometer. The enzyme activity was expressed as Units per mg tissue protein [35].

Catalase (CAT)

Catalase was assayed by measuring the breakdown of hydrogen peroxide (H₂O₂) according to the method of Beers and Sizer. The weighed amounts of the hepatic, heart, kidney and spleen tissues were homogenized in 5% ice-cold 50 mM phosphate buffer pH 7.2. The homogenates were then centrifuged at 12,000 g for 12 min. The supernatant, thus obtained, was then carefully collected and incubated with 0.01 ml of absolute ethanol at 4 °C for 30 min. Thereafter, 10% Triton X-100 was added to have a final concentration of 1%. The resulting sample was used to determine the CAT activity by measuring the breakdown of H₂O₂ spectrophotometrically at 240 nm. The enzyme activity was expressed as micromoles of H₂O₂ consumed per min per mg protein [36].

Expression of Cox-2 in synovial tissue

Expression of cyclooxygenase-2 (cox-2) in synovial tissues was determined by immunoblotting. Protein levels in the

tissue homogenates were determined by the Bradford method. Twenty micrograms of each sample was electrophoresed on polyacrylamide gel and transferred onto a nitrocellulose membrane. After blocking with 7% skimmed milk, the blots were incubated overnight at 4 °C with primary antibodies against cox-2 (1:1000; Chemicon, Temecula, CA, USA). After extensive washes in PBS-Tween, blots were incubated with appropriate secondary antibodies conjugated with peroxidase (Vector Laboratories). The blots were again washed in PBS-Tween and processed for development using chemiluminescence reagent (Millipore, Billerica, MA, USA). The images were captured and analysed using Chemigenius, Bioimaging System (Syngene, Cambridge, UK). The blots were then stripped (30 min at 50 °C in 62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, and 100 mM β-mercaptoethanol) and reprobed with anti-β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to determine equivalent loading of samples [37].

Statistical analysis

One-way model 1 ANOVA (Analysis of Variance) was performed between the groups. In ANOVA, observed variance is partitioned into components due to different explanatory variables. A level of $P < 0.05$ or $P < 0.001$ was considered significant. Significant differences of the means between the groups were performed by one-way ANOVA. Scheffe's *F*-test had been performed as *post hoc* test for multiple comparisons of means of different groups when significant *F* value was found [38].

Results

Recovery of bacteria from blood, spleen and synovial tissue and induction of arthritis after single *in vivo* injection of *S. aureus* (AG-789) followed by gentamicin and ascorbic acid after an interval of 2 h, respectively

In isolates obtained 3 days after the start of the infection, growth of *S. aureus* was noted not only in the joints but also in the blood and spleen. The induction of clinical arthritis when calculated from the mean diameters of the wrist and ankle joints of the mice, it was observed that there was 59% induction of clinical arthritis in the *S. aureus*-infected group compared with the control group. Bacterial burden in blood, spleen and synovial tissues was still prominent at 9 days post-infection (dpi), although no detectable amount of bacteria was found in either blood, spleen or synovial tissue at 15 dpi. However, treatment of mice with gentamicin after infection followed by ascorbic acid eradicates the bacteria from the blood and also significantly reduces the bacterial burden in both spleen and synovial tissue at 3 dpi and also showed reduced swelling of arthritis ($P < 0.05$) (Table 1). Combined

Table 1 Recovery of bacteria from blood, spleen and synovial tissue and induction of arthritis after intravenous injection of *S. aureus* (AG-789) followed by treatment with gentamicin alone or in combination with ascorbic acid..

Groups	Days after infection	No. of bacterial colonies (Mean $\pm$ SD)			
		Spleen (per g of tissue)	Synovial tissue (per gm of tissue)	Blood (per ml of blood)	Induction of arthritis (%)
	3 dpi ^a				
Control	3	0	0	0	0
<i>S. aureus</i>	3	3500 $\pm$ 57.74	3500 $\pm$ 57.74	690 $\pm$ 17.32	59.17
<i>S. aureus</i> + gentamicin	3	2017 $\pm$ 28.87	1920 $\pm$ 11.55	280 $\pm$ 20	26.49
<i>S. aureus</i> + ascorbic acid	3	1281 $\pm$ 30.38	1710 $\pm$ 40.41	260 $\pm$ 11.55	35.31
<i>S. aureus</i> + gentamicin + ascorbic acid	3	941 $\pm$ 34.3	57 $\pm$ 1.15	85 $\pm$ 5	20.29
Gentamicin alone	3	Not detectable			
Ascorbic acid alone	3	Not detectable			
	9 dpi				
Control	9	0	0	0	
<i>S. aureus</i>	9	713 $\pm$ 23.96	781 $\pm$ 11.97	70 $\pm$ 5.77	28.60
<i>S. aureus</i> + gentamicin	9	150 $\pm$ 20.41	212 $\pm$ 7.22	50 $\pm$ 5.77	13.86
<i>S. aureus</i> + ascorbic acid ^b	9	175 $\pm$ 14.4	218 $\pm$ 6.25	55 $\pm$ 5	16.07
<i>S. aureus</i> + gentamicin + ascorbic acid	9	162 $\pm$ 12.5	81 $\pm$ 6.25	25 $\pm$ 5	0.68
Gentamicin alone	9	Not detectable	Not detectable		
Ascorbic acid alone	9	Not detectable	Not detectable		

No mentionable bacterial colonies were detected at 15 dpi in any of the above groups

Blood and organs were removed and processed as detailed in materials and methods. Numbers of bacterial cells in blood, spleen and synovial tissues were determined on day 3, 9 and 15 post-infections. Each result is the mean and standard deviation for a group of six mice.

aNumber of bacterial CFU in blood, spleen and synovial tissues at 3 dpi are significant at $P < 0.05$.

bS. *aureus* alone versus *S. aureus* + Ascorbic acid at 9 dpi; ($P > 0.05$, non-significant).

treatment of mice with gentamicin and ascorbic acid also reduced the bacterial density in blood and organs at 9 dpi with less induction of clinical arthritis (Table 1).

Experimental evaluation of arthritis: effect of gentamicin alone or in combination with ascorbic acid on *S. aureus* (AG-789) infection-induced swelling of joints

The result showed that there was significantly increased swelling of wrist and ankle joints in pathogenic strain *S. aureus* (AG-789)-infected mice after 3, 9 and 15 days of infection compared with uninfected control group ($P < 0.05$). Administration of gentamicin after *S. aureus* infection followed by ascorbic acid treatment showed significant reduction in the swelling of joints at day 3 when compared with *S. aureus* alone infected mice (Table 2). Treatment of mice with gentamicin alone or in combination with ascorbic acid after *S. aureus* infection showed significant gross reduction in clinical arthritis as compared with *S. aureus*-induced arthritis at 15 dpi (Table 2).

Serum levels of TNF- α , IFN- γ , IL-6 and IL-10 of different groups of mice at 3, 9 and 15 days post-infection

Serum TNF- α , IFN- γ , IL-6 levels but not IL-10 were increased significantly after *S. aureus* infection ($P < 0.05$). Treatment of mice with gentamicin alone or in combination with ascorbic acid after infection could able to

significantly downregulate the serum TNF- α , IFN- γ , IL-6 at day 3, 9 and 15. However, gentamicin alone or in combination with ascorbic acid could also increase the serum IL-10 significantly on day 3, 9 and 15 (Fig. 1).

Synovial tissue myeloperoxidase (MPO) enzyme activity

The activity of MPO enzyme which is an indicator for neutrophil infiltration from the synovial tissue homogenate it was observed that, on 3, 9 and 15 days post-infection, synovial tissue MPO enzyme activity was significantly higher at 9 dpi for the pathogenic strain-AG-789 than the uninfected control group. When gentamicin was administered alone or in combination with ascorbic acid, it caused significant ($P < 0.05$) reduction in tissue MPO enzyme activity in the case of the pathogenic strain-AG-789-infected group only at day 3, 9 and 15 after infection (Table 3).

Effect of Gentamicin and ascorbic acid treatment on synovial tissue Cyclooxygenase-2 level in the *S. aureus*-infected mice

Immunoblot analysis of synovial tissue homogenate showed that COX-2 level was significantly increased at 3 days post-infection (dpi) in case of the AG-789 *S. aureus*, which was gradually decreased at 9 and 15 dpi. After treatment with gentamicin along with ascorbic acid, cox-2 level was significantly decreased on day 15 (Fig. 2).

Table 2 Experimental evaluation of arthritis: effect of gentamicin alone or in combination with ascorbic acid on *S. aureus* (AG-789) infection induced swelling of joints..

Groups	No. of mice	No. of mice died	No. of mice showing clinical signs of arthritis	Swelling of wrist joints ^a in mm (Mean $\pm$ SE)			Swelling of ankle joints ^b in mm (Mean $\pm$ SE)		
				Day 3	Day 9	Day 15	Day 3	Day 9	Day 15
Control	4	0	0	4.11 $\pm$ 0.006	6.697 $\pm$ 0.154	8.49 $\pm$ 0.081	8.21 $\pm$ 0.006	8.047 $\pm$ 0.026	10.57 $\pm$ 0.15
<i>S. aureus</i>	6	0	6	9.24 $\pm$ 0.75	8.433 $\pm$ 0.378	10.08 $\pm$ 0.016	10.37 $\pm$ 0.151	10.523 $\pm$ 0.211	12.48 $\pm$ 0.03
<i>S. aureus</i> + gentamicin	6	0	6	6.463 $\pm$ 0.154	8.25 $\pm$ 0.05	9.93 $\pm$ 0.038	9.12 $\pm$ 0.319	8.533 $\pm$ 0.15	11.57 $\pm$ 0.27
<i>S. aureus</i> + ascorbic acid	6	0	6	7.683 $\pm$ 0.543	8.4 $\pm$ 0.066	9.99 $\pm$ 0.032	8.99 $\pm$ 0.433	8.71 $\pm$ 0.27	11.76 $\pm$ 0.218
<i>S. aureus</i> + gentamicin + ascorbic acid	6	0	6	6.193 $\pm$ 0.054	6.41 $\pm$ 0.147	8.822 $\pm$ 0.042	8.627 $\pm$ 0.082	8.39 $\pm$ 0.082	10.83 $\pm$ 0.026
Reduction of arthritis (in percentage) when compared with <i>S. aureus</i> alone versus <i>S. aureus</i> + gentamicin + ascorbic acid group at 3, 9 and 15 dpi				33.05	23.98	12.48	16.81	20.27	13.22

The severity of arthritis in each wrist and ankle joints was quantified daily by an experimental assessment of swelling of the joint tissues as described in the methods section. Measurements were performed with a dial gauge caliper in millimeters. Swelling of wrist and ankle joints (in mm $\pm$ SD) in mice was shown. The difference was statistically significant ($P < 0.05$).

aSwelling of wrist joint at 3 and 15 dpi; *S. aureus* (AG-789) versus *S. aureus* + ascorbic acid not significant at $P > 0.05$.

bSwelling of ankle joint at 3, 9 and 15 dpi; all groups were significant at $P < 0.05$.

Effects of gentamicin–ascorbic acid co-therapy against *S. aureus* infection-induced oxidative stress in heart, liver, spleen and kidney tissues of mice and its relation with progression of septic arthritis

Treatment of mice with *S. aureus* caused elevation of LPO in all the tissues tested, viz., heart (Fig. 3A), liver (Fig. 4A), kidneys (Fig. 5A) and spleen (Fig. 6A). In all the tissues, the elevation of LPO was found to be significantly higher 3 and 9 days post-infection except liver. However, this elevation in LPO was found to be significantly ameliorated when the mice were co-treated with gentamicin and ascorbic acid in combination indicating efficacy of the combination in the abatement of the oxidative stress induced in the tissue due to the bacterial infection.

That oxidative stress is induced following *S. aureus* infection is also evident from our finding that the level of reduced glutathione (GSH) was found to be decreased in all the tissues tested in mice. However, when the mice were co-treated with gentamicin and ascorbic acid in combination, the tissue glutathione level in most of the cases was found to be restored to near control values (Heart – Fig. 3B, Liver – Fig. 4B, Kidney – Fig. 5B and Spleen – Fig. 6B). These results indicate that the antibiotic–anti oxidant combination is capable of ameliorating the oxidative stress induced in these organs due to *S. aureus* infection.

Similarly, we have examined the activities of the key antioxidant enzymes like SOD and CAT in all the four tissues tested. The results indicate that SOD activity in

S. aureus-infected group increased significantly in heart (Fig. 3C), liver (Fig. 4C) and spleen tissues (Fig. 6C). However, the SOD activity was decreased and almost restored to control in combination with gentamicin and ascorbic acid in heart (Fig. 3C), liver (Fig. 4C), and spleen (Fig. 6C) tissues at 3 days post-infection, whereas in kidney (Fig. 5C), the SOD activity was significantly decreased in the *S. aureus*-treated group at days 3, 9 and 15 post-infection and was found to be restored to control in combination with gentamicin and ascorbic acid at days 3 and 15 post-infection. The SOD activity of the heart (Fig. 3C) and liver (Fig. 4C) tissue at day 9 post-infection was found to be increased in the *S. aureus*-treated group, which was found to be almost near the control level by the combination in heart tissue but increased further in liver tissue. However, at day 15 post-infection, the SOD activity was found to be decreased in all the tissues except liver where it increased in the *S. aureus*-treated group. However, in kidney (Fig. 5C), heart (Fig. 3C) and splenic tissues (Fig. 6C), significant increase was found in the gentamicin and ascorbic acid combination group at day 15 post-infection. The improvement in hepatic SOD activity was significant but did not reach the control level. The results further indicated that the combination of gentamicin and ascorbic acid was able to restore the activity of the enzyme in all the four tissues. However, the level of activity of SOD was found to be considerably higher in all the groups of mice tissues 15 days post-infection. But, SOD activity remained inhibited in *S. aureus*-infected group in kidney and spleen tissues at day 9 post-infection.

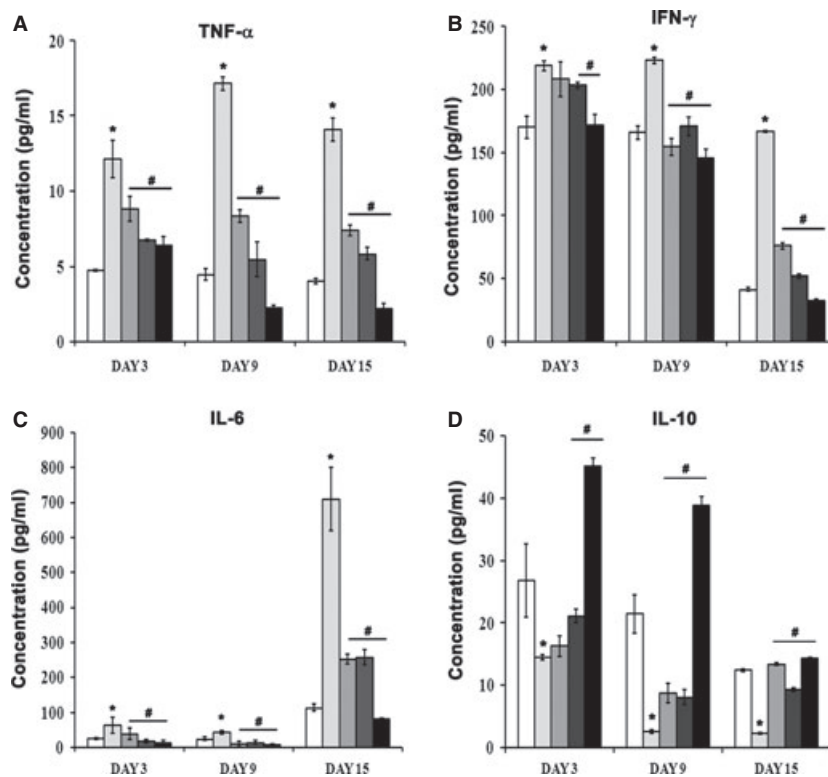


Figure 1 Serum levels of TNF- α , IL-6, IFN- γ and IL-10 in different groups of mice at 3, 9 and 15 days post-infections. Levels of TNF- α (A), IFN- γ (B), IL-6 (C) and (D) IL-10 in serum from *S. aureus*-infected mice treated with gentamicin alone or in combination with ascorbic acid after Staphylococcal infection and from non-infected control animal were determined by utilizing ELISA according to the manufacturer's recommendations and were expressed from triplicate experiments. Without infection control versus *S. aureus* AG-789 alone, significant increase in TNF- α and IFN- γ and IL-6 but decrease in IL-10, * $P < 0.05$; *S. aureus* AG-789 alone versus *S. aureus* AG-789 + gentamicin, significant decrease in TNF- α , IFN- γ and increased IL-10, # $P < 0.05$, *S. aureus* AG-789 alone versus *S. aureus* AG-789 + gentamicin + ascorbic acid, significant decrease in TNF- α , IFN- γ and increased IL-10, ## $P < 0.05$.

Table 3 MPO activity of synovial tissue of mice after intravenous injection of *S. aureus* (AG-789) followed by treatment with gentamicin alone or in combination with ascorbic acid at day 3, 9 and 15 post-infection..

Groups	Change in MPO activity/min/mg of protein		
	Day 3 post-infection	Day 9 post-infection	Day 15 post-infection
Control	0.062 $\pm$ 0.0055	0.095 $\pm$ 0.004	0.064 $\pm$ 0.0009
<i>S. aureus</i>	0.11 $\pm$ 0.0058	0.144 $\pm$ 0.024	0.093 $\pm$ 0.0062
<i>S. aureus</i> + gentamicin	0.027 $\pm$ 0.0021	0.066 $\pm$ 0.009	0.048 $\pm$ 0.0033
<i>S. aureus</i> + ascorbic acid	0.054 $\pm$ 0.0058	0.1 $\pm$ 0.031	0.051 $\pm$ 0.0034
<i>S. aureus</i> + gentamicin + ascorbic acid	0.071 $\pm$ 0.005	0.1 $\pm$ 0.032	0.072 $\pm$ 0.0022

MPO activity was analysed as index of neutrophil infiltration in the synovial tissue. The rate of change in absorbance was measured spectrophotometrically at 405 nm. MPO activity has been defined as the concentration of enzyme degrading 1 μ mol of peroxide/min at 37 °C and was expressed as change in absorbance/min.mg of protein. The results were reproduced in three repeated experiments. Data are expressed as mean $\pm$ sd of mice per group. All the values are significant in the population mean ($P \leq 0.05$).

The activity of CAT, another important antioxidant enzyme, was found to be increased in heart (Fig. 3D), liver (Fig. 4D) and kidney tissues (Fig. 5D), while the activity was found to be decreased in mouse spleen (Fig. 6D) following infection with *S. aureus* at day 3, 9 and 15 post-infection. However, the CAT activity was found to be significantly decreased in liver, kidney and spleen tissues in combination with gentamicin and ascorbic acid at 3, 9 and 15 days post-infection except liver at day 15 and restored near the control level at day 3 post-infection. However, in the spleen tissue, the CAT activity was decreased significantly following *S. aureus* infection at days 3, 9 and 15 post-infection and increased significantly when the mice

were treated with the combination of gentamicin and ascorbic acid at 3, 9 and 15 days post-infection and restored to near normal at day 3 post-infection. The CAT activity was found to be otherwise prominent in 15 days post-infection, particularly in kidney and heart. This may be an adaptive response.

Discussion

Septic arthritis is triggered by the haematogenous spread of bacteria from an initial nidus to the joint spaces. We studied the pathogenesis of *S. aureus* septic arthritis in a murine model in which bacteria are injected intravenously,

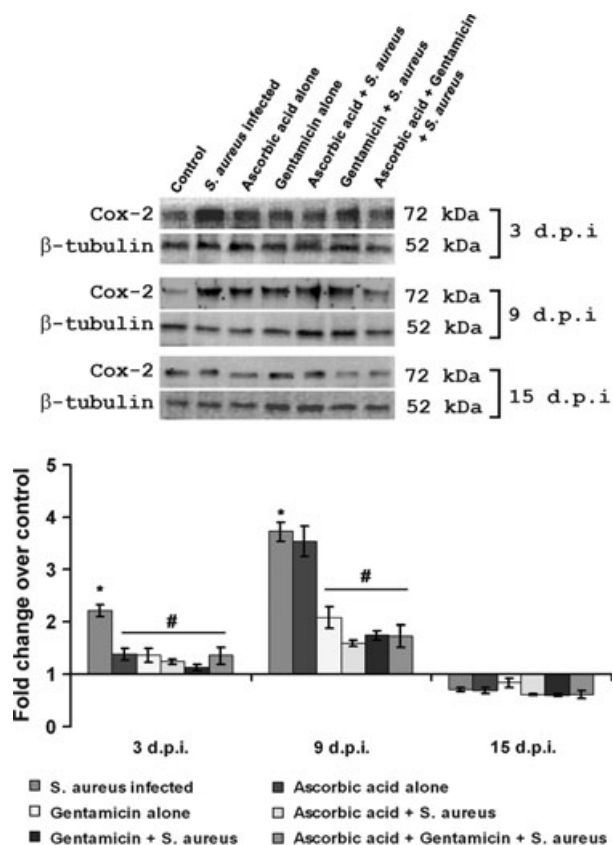


Figure 2 Expression of Cox-2 after treatment with gentamicin in combination with ascorbic acid in synovial tissue. Expression of COX-2 was measured in terms of fold change over control. Highest level of COX-2 was found on day 9 post-infection. Reduction in COX-2 level was found after treatment with gentamicin alone or in combination with ascorbic acid at 3 and 9 dpi. Control versus pathogenic strain *S. aureus* AG-789 at 3 and 9 dpi (* $P < 0.01$ with respect to control, *S. aureus* AG-789 alone versus *S. aureus* + gentamicin, *S. aureus* + ascorbic acid, *S. aureus* + gentamicin + ascorbic acid (# $P < 0.01$ with respect to 3 and 9 dpi-infected sample).

seed to the joints and cause the disease that clinically resembles septic arthritis in humans.

There is a close correlation between clinical arthritis and the presence of bacteria in the synovial tissue at 3 and 9 dpi. Typically, the peak of bacterial burden after intravenous staphylococcal inoculation occurs in blood and tissue within the first 3 dpi as evident from the CFU count with 59.17% induction of clinical arthritis in the infected group (Table 1), whereas only 20.29% of arthritic when infected mice are treated with gentamicin along with ascorbic acid indicating the effectiveness of combined treatment in reducing clinical manifestations of arthritis. This type of haematogenously spread joint infection is by far the most common finding in human staphylococcal arthritis, including homing of bacteria to the joints and exposure to the innate immune system would be bypassed [39].

To cause disease, bacteria have to survive in blood, spread from the blood stream to the joint and survive there,

at least for a while (3 dpi). It has been shown previously that the degree of arthritis and inflammation under normal circumstances is dependent on the amount of bacteria injected and the ability of the host to clear the bacteria.

Furthermore, because in some previous experiments, it was seen at very low doses of gentamicin that gentamicin was concentrated within phagosomes. When tested in the presence of gentamicin, cytokines thought to induce bactericidal activity may instead only slowed phagosomal escape or increased pinocytosis, thus increasing gentamicin-mediated killing of intracellular bacteria, suggesting that even small amounts of gentamicin could kill intracellular *L. monocytogenes*. Furthermore, gentamicin could cause non-bactericidal macrophages to appear to be bactericidal [40]. Effect of gentamicin on intracellular killing of *S. aureus* was also reported [41] and supporting our observation.

We found that early administration of antibiotic alone or in combination with ascorbic acid eradicates the bacteria from the blood and tissue and also correlated with joint inflammation. Although there are no detectable bacterial colonies in either blood, spleen or synovial tissues at 15 dpi, still mice showed signs of arthritis, suggesting swelling not be directly due to the bacterial burden in the blood or tissues but might be due to the coagulase induced during the course of infection. Therefore, the occurrence, even in the treated mice, is not surprising, bearing in the mind that treatment was started 24 h after inoculation of bacteria. This was performed in an attempt to reflect the clinical situation when patients present with staphylococcal infections. The ascorbic acid was administered after 2 h of antibiotic treatment. Owing to the fact that there is such a short time difference between ascorbic acid and antibiotic treatment, it may be questioned whether this has any physiological significance. It was reported that sublethal *E. coli* endotoxin increases ascorbate recycling in liver and ascorbate concentration in liver, kidney, adrenal gland and heart at 3 h post-injection [17] also supported our study. This indicates that pharmacologically administered ascorbate may improve the overall antioxidant capacity of the tissues, which helps prevent the changes in the antioxidant status from occurring following infection with *S. aureus*. Thus, the focus should be on the effect, rather than the time schedule. If the drug is found to be effective, then its effect may extend up to later time periods.

Among the oxidative stress sensitive generated molecules, cytokines are pivotal ROS mediators that act in synergistic or additive manner. Therefore, ROS and cytokines might be integrated in arthritis and are engaging in cross-talking in almost every organ like heart, liver, spleen and kidney. In this study, IFN- γ , TNF- α and IL-6 production was increased initially within 3 days post-infection and decreased thereafter following treatment with gentamicin and ascorbic acid. Therefore, it is likely that increased IFN- γ released into the circulation in the first phase (3 dpi) by the

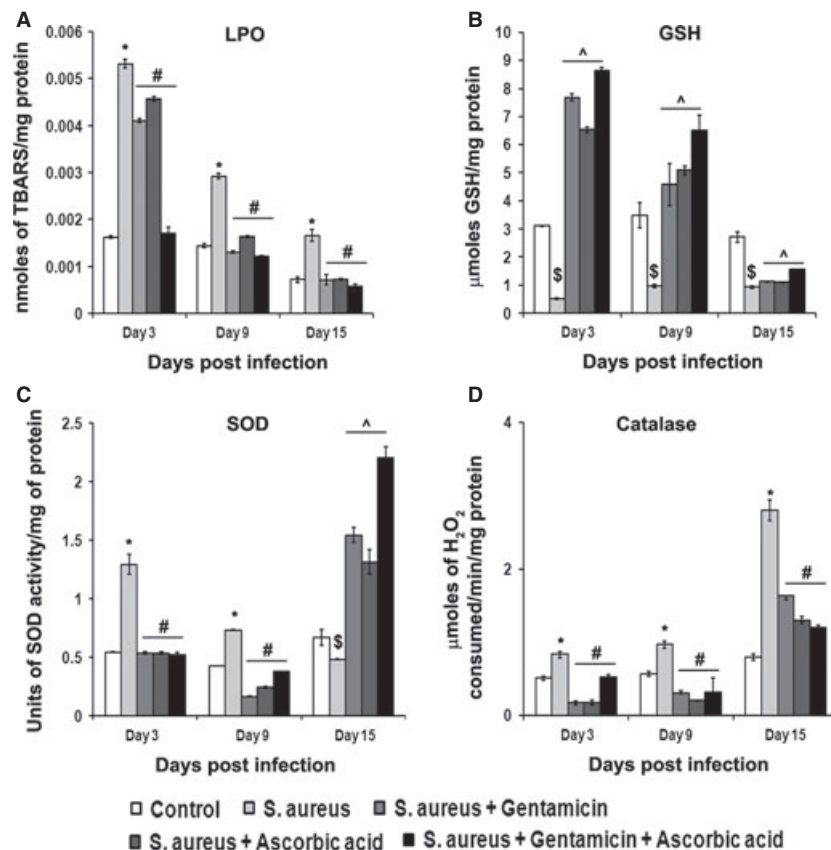


Figure 3 Alteration in mouse cardiac antioxidant status like reduced glutathione level and the activities of antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT). (A) lipid peroxidation level, without infection control versus *S. aureus* alone, significant increase at 3, 9, 15 dpi $*P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin, significant decrease at 3, 9 and 15 dpi $\#P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant decrease at 3, 9 and 15 dpi $\$P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin, significant increase at 3, 9 and 15 dpi $^{\wedge}P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant increase at 3, 9, 15 dpi $^{\wedge}P < 0.05$. (B) GSH, without infection control versus *S. aureus* alone, significant decrease at 3, 9 and 15 dpi $\$P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin, significant increase at 3, 9 and 15 dpi $^{\wedge}P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant increase at 3, 9, 15 dpi $^{\wedge}P < 0.05$. (C) SOD, without infection control versus *S. aureus* alone, significant increase at 3, 9 dpi $*P < 0.05$ but decreased significantly at 15 dpi $\$P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin, significant decrease at 3 and 9 dpi $\#P < 0.05$ but significant increase at 15 dpi $^{\wedge}P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant increase at 15 dpi $^{\wedge}P < 0.05$. (D) CAT, without infection control versus *S. aureus* alone, significant increase at 3, 9 and 15 dpi $*P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant decrease at 3, 9, 15 dpi $\#P < 0.05$.

administration of 5×10^7 CFU of *S. aureus* cells or their exotoxins demonstrated a detrimental effect on the host. We found that severity of arthritis is associated with altered balance of inflammatory cytokines, and conversely, altering the balance of inflammatory cytokines has a significant impact on the severity of arthritis.

Of several septic arthritis-related molecular pathways with anti-inflammatory actions, we chose to focus on IL-10 as a representative of cytokine in this class. In serum, which reflects the primary site of inflammation in this model, IL-10 continues to increase even at 15 dpi after treatment of mice with gentamicin and ascorbate. This IL-10 level increment dictates the resolution of inflammation and may be a positive prognostic indicator for recovery of arthritis due to the combined therapy. IL-10 inhibits the production of reactive oxygen and reactive nitrogen intermediates when monocyte/macrophages are activated by IFN- γ and therefore may be important in determining the outcome of

arthritis. As lack of IL-10 causes impaired clearance of bacteria leading to a more destructive cause of arthritis, therefore, this elevated IL-10 in the antibiotic-antioxidant combined treated mice might be essential for efficient elimination of bacteria and therapy for protection against septic arthritis.

Our studies clearly indicate involvement of oxidative stress in all the tissues tested in mouse models following infection with *S. aureus*. Treatment of mice with gentamicin alone, though effective in killing bacteria, seemed not efficient in reducing oxidative stress status neither ameliorating inflammation of the joints. However, when the mice were co-treated with gentamicin and ascorbic acid, the oxidative stress status of the liver, kidneys, spleen and heart was found to significantly decrease. This is reflected in the amelioration of alterations in the levels of tissue LPO and GSH as well as the activities of the key antioxidant enzymes like SOD and CAT. *S. aureus* infection-induced GSH

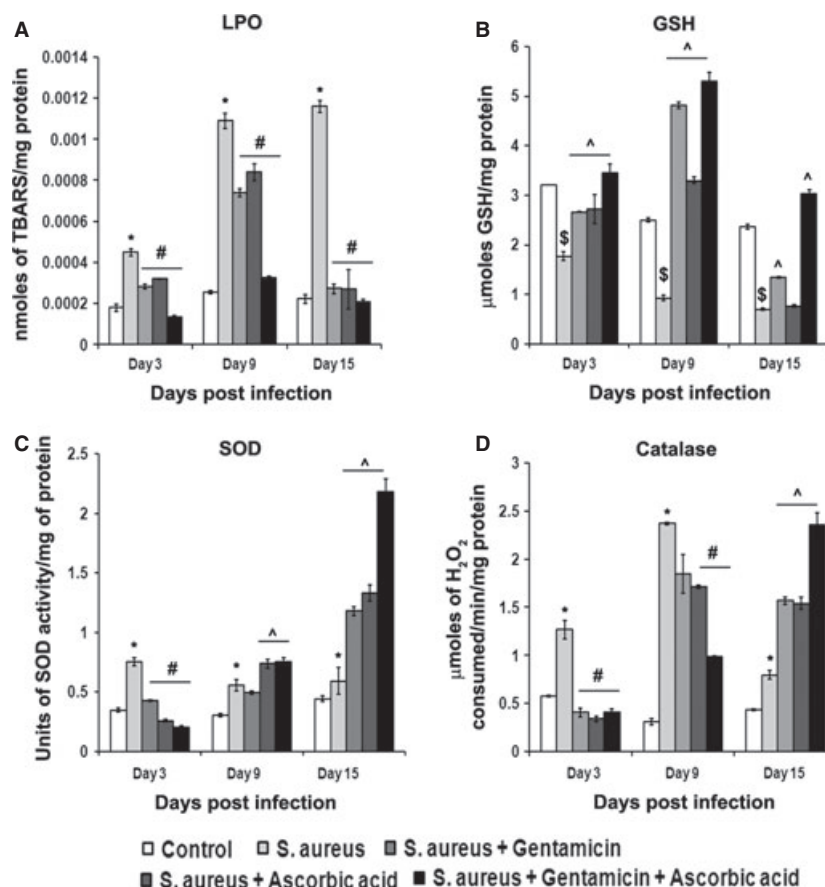


Figure 4 Alteration in mouse hepatic antioxidant status like reduced glutathione level and the activities of the antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT). (A) lipid peroxidation level, without infection control versus *S. aureus* alone, significant increase at 3, 9, 15 dpi * $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin, significant decrease at 3, 9 and 15 dpi # $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant decrease at 3, 9 and 15 dpi # $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin, significant increase at 3, 9, and 15 dpi ^ $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant increase at 3, 9, 15 dpi ^ $P < 0.05$. (B) GSH, without infection control versus *S. aureus* alone, significant decrease at 3, 9 and 15 dpi \$ $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin, significant increase at 3, 9, and 15 dpi ^ $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant increase at 3, 9, 15 dpi ^ $P < 0.05$. (C) SOD, without infection control versus *S. aureus* alone, significant increase at 3, 9 and 15 dpi * $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant decrease at 3 dpi # $P < 0.05$; but significant increase at 9 and 15 dpi ^ $P < 0.05$. (D) CAT, without infection control versus *S. aureus* alone, significant increase at 3, 9 and 15 dpi * $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant decrease at 3 and 9 dpi # $P < 0.05$; but significant increase at 15 dpi ^ $P < 0.05$.

depletion may be associated with augmentation of an oxidative stress-mediated production of proinflammatory cytokines particularly TNF- α and IFN- γ because GSH is reported to play an important role in the polarization of TH1/TH2 balance [42]. Alteration in the activities of antioxidant enzymes in different tissues tested in our case may be due to the differences in the infiltrated bacteria and the laboratory animals because it is reported that the capacity of antioxidant enzyme induction to ROS is different from species to species [43]. Typically, the peak bacterial burden after i.v. staphylococcal inoculation occurs in blood within first 3 days and may be somewhat latter in kidneys, heart, liver which we have not been tested in our case. These tissue-fixed bacteria may lead to enhanced ROS production, which could be downregulated after combined treatment by inducing SOD and CAT in these tissues. The non-phagocytosed, tissue bound bacteria and, more impor-

tantly, their exotoxins activate macrophages and monocytes to release proinflammatory cytokines like TNF- α , IFN- γ , IL-1 which enter the circulation and trigger a general inflammatory response. Moreover, further studies are required particularly malondialdehyde, indicator of lipid peroxidation, level must be measured and pharmacokinetics of gentamicin and tissue ascorbic acid content after single daily dose or multiple daily dose of antibiotic/antioxidant to be determined in arthritic mice. Therefore, determining the cardiac, renal, splenic and hepatic gentamicin concentration along with tissue ascorbate level at 3, 9 and 15 dpi will be helpful to figure out the effect of combined treatment on tissue-specific alteration in antioxidant enzyme activities in *S. aureus* infection-induced arthritis.

It was reported that extracellular cytochrome c triggers sinusitis in a healthy host and might therefore play a role in the pathogenesis of arthritis. The sinusitis triggered by

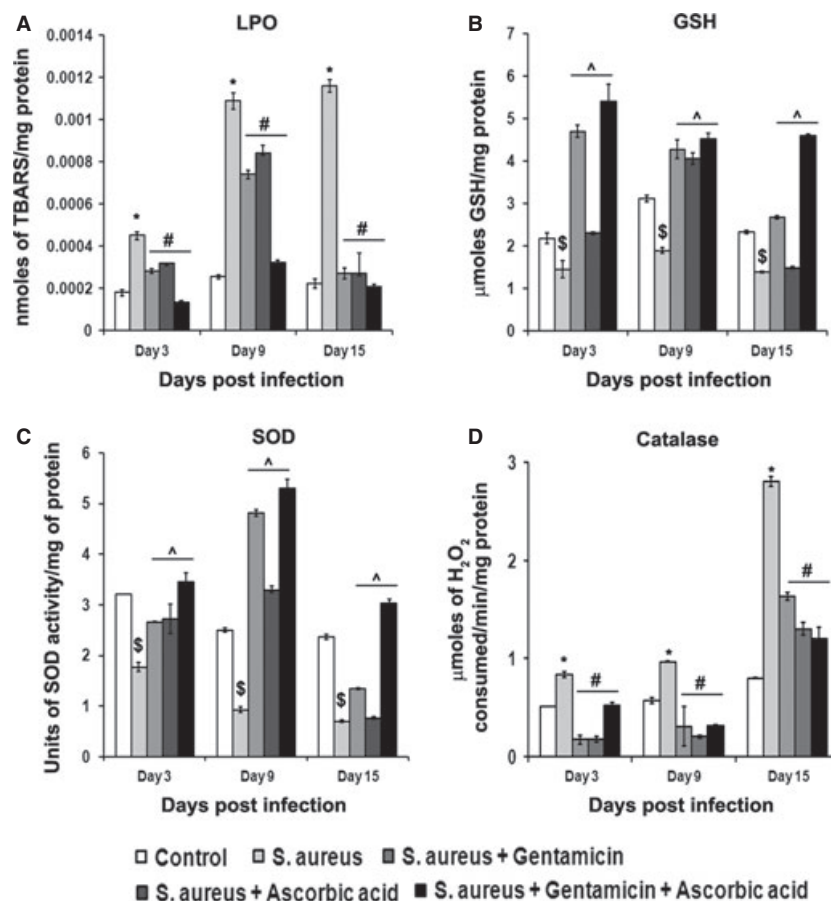


Figure 5 Alteration in the antioxidant status like reduced glutathione level and the activities of the antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) in mouse kidney. (A) lipid peroxidation level, without infection control versus *S. aureus* alone, significant increase at 3, 9, 15 dpi * $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin, significant decrease at 3, 9 and 15 dpi # $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant decrease at 3, 9 and 15 dpi # $P < 0.05$. (B) GSH, without infection control versus *S. aureus* alone, significant decrease at 3, 9 and 15 dpi \$ $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin, significant increase at 3, 9 and 15 dpi ^ $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant increase at 3, 9, 15 dpi ^ $P < 0.05$. (C) SOD, without infection control versus *S. aureus* alone, significant decrease at 3, 9 and 15 dpi \$ $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant increase at 3, 9 and 15 dpi ^ $P < 0.05$. (D) CAT, without infection control versus *S. aureus* alone, significant increase at 3, 9 and 15 dpi * $P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant decrease at 3, 9 and 15 dpi # $P < 0.05$.

cytochrome c was rather short-lasting by day 10 the incidence of arthritis had decreased, indicating lack of acquired immunity as a driving force. It is due to this that the recovery in 15 days post-infection in most of the tissues studied in our model was found to be higher. This may also be due to the adaptive responses wherein tissues maintain their metabolic status with minimum damage despite the presence of chronic oxidative stress. Reduction in oxidative stress status due to co-therapeutic approach may also reduce the ROS burden in the tissues tested which may have protective action on the mitochondrial membrane thereby preventing the cytochrome c leakage [44]. This may facilitate tissue repair mechanisms to override upon the destructive mechanisms due to oxidative stress following *S. aureus* infection in our mouse model. Endogenous ascorbic acid has also been shown to protect rodent hepatocytes from lethal oxidative stress [17]. Ascorbic acid

is involved in the synthesis of intracellular substances such as collagen fibres found in various forms of connective tissues and matrix of the bone. It is highly concentrated in leucocytes and is used rapidly during infection to prevent oxidative damage. Therefore, additional ascorbic acid treatment or multiple daily dose of ascorbic acid is required during arthritis and tissue regeneration.

Oxidative stress-induced inflammation and tissue damage in septic arthritis is a problem of global concern. Infection in the joints and synovial fluid further aggravate the situation. Resistance to antibiotic therapy causes the situation to worsen even more leading to oxidative damage. The ascorbic acid along with gentamicin treatment reduces the oxidative stress in various organs and improves the outcome of the infection. The success of this co-therapeutic approach in our mouse model seems to have future therapeutic relevance.

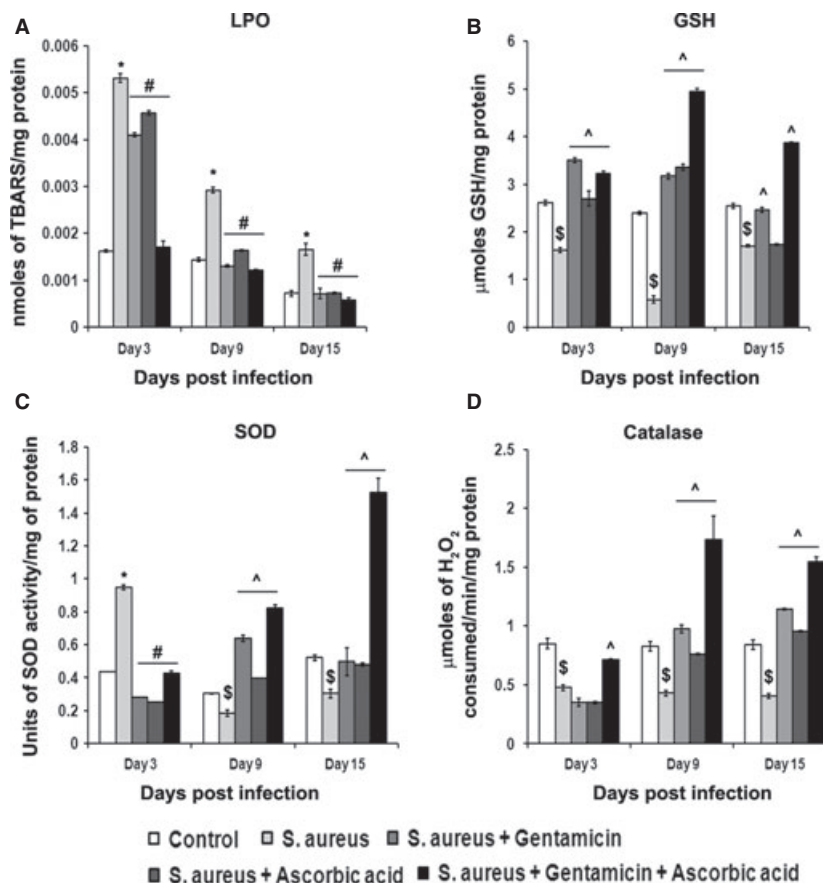


Figure 6 Alteration in the antioxidant status like reduced glutathione level and the activities of the antioxidant enzymes such as superoxide dismutase (SOD) and catalase splenic tissue of mice. (A) lipid peroxidation level, without infection control versus *S. aureus* alone, significant increase at 3, 9, 15 dpi $*P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin, significant decrease at 3, 9 and 15 dpi $\#P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant decrease at 3, 9 and 15 dpi $\$P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin, significant increase at 3, 9, and 15 dpi $\wedge P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant increase at 3, 9, 15 dpi $\wedge P < 0.05$. (B) GSH, without infection control versus *S. aureus* alone, significant decrease at 3, 9, and 15 dpi $\$P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin, significant increase at 3, 9, and 15 dpi $\wedge P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant increase at 3, 9, 15 dpi $\wedge P < 0.05$. (C) SOD, without infection control versus *S. aureus* alone, significant increase only at 3, dpi $*P < 0.05$ but decrease significantly at 9 and 15 dpi $\$P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant decrease only at 3 dpi $\#P < 0.05$, but significant increase at 9 and 15 dpi $\wedge P < 0.05$. (D) Catalase, without infection control versus *S. aureus* alone, significant decrease at 3, 9 and 15 dpi $\$P < 0.05$; *S. aureus* alone versus *S. aureus* + gentamicin + ascorbic acid, significant increase at 3, 9 and 15 dpi $\wedge P < 0.05$.

Conclusion

Although staphylococcal septic arthritis disease whose profiles suggest several virulence components and adapting new protocols like antibiotics plus antioxidants to combat septic arthritis is clearly a priority, the underlying mechanisms are not known. The purpose of this study was to investigate the efficacy of the treatment with gentamicin alone or in combination with ascorbic acid on the *S. aureus* infection-induced arthritis in a mouse model and its underlying mechanisms. Finally, we consider the present co-supplementation interventions in animals as preventive, but not therapeutic, because it is able to maintain the normal function and redox status of the body cells from a general population of mice.

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