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Ampicillin alone and in combination with riboflavin modulates *Staphylococcus aureus* infection induced septic arthritis in mice

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Effects of ampicillin (Amp) in combination with riboflavin on septic arthritis in mice infected with *Staphylococcus aureus* have been reported. Ampicillin was given at 100 mg/kg after 24 h of infection, followed by riboflavin (Ribo) at 20 mg/kg body wt, after 2 h of Amp treatment. Mice were sacrificed at 3, 9, 15 days post infection (dpi). Combined treatment of infected mice with ampicillin and riboflavin eradicated the bacteria from blood, spleen and synovial tissue and showed a significant gross reduction in arthritis, reduced serum levels of TNF-α and IFN-γ. *S. aureus* infected mice exhibited higher synovial TNF-α and IL-6, which was also reduced by ampicillin and riboflavin treatment. *S. aureus* infected mice showed a disturbed antioxidant status measured in terms of cellular anti-oxidants like reduced glutathione and anti-oxidant enzymes such as superoxide dismutase and catalase and were ameliorated when the animals were co-treated with ampicillin along with riboflavin. Results of the study showed that combined treatment with anti-oxidant and antibiotic may protect from staphylococcal arthritis and may ameliorate oxidative stress caused by *S. aureus* infection.

Keywords: Ampicillin, Antioxidant enzymes, Cytokines, Inflammation, Reactive oxygen species, Riboflavin, *S. aureus*, Septic arthritis, Synovial inflammation, Toxic shock syndrome toxin

Staphylococcus aureus (*S. aureus*) is the most frequently isolated bacterial species associated with septic arthritis patients and accounts for 8-16% of the cases[1] and approximately 75% of the cases with rheumatoid arthritis[2]. Intravenous inoculation of mice with an exotoxin (TSST-1) producing strain of *S. aureus* leads to the development of severe septic arthritis[3,4]. It was shown that staphylococcal products together with post inflammatory mediators leading to cartilage destruction despite antibiotic treatment[5]. Role of reactive oxygen species (ROS) in immunopathogenesis of rheumatoid arthritis has been clarified[6]. Increased ROS have been documented at sites of inflammation such as synovial joints of patients with inflammatory arthritis[7]. However, the role of ROS in initiation and progression of synovial inflammation in arthritis has not been studied extensively[8]. Recent evidences implicated that intracellular ROS production plays a key role in modulation of the release of other mediators of inflammation[9]. Studies have suggested that inflammation and cytotoxicity occurs when an imbalance exists between ROS production and antioxidant enzyme activity[10]. Balance between ROS generation and anti-oxidant defenses are thus important and help to prevent the onset of chronic disease[11]. Hemodynamic changes in sepsis are mediated by production of pro-inflammatory cytokines and by the overproduction of free radicals and oxidant mediators of septic arthritis[12].

It is necessary to adopt new methods to treat ongoing infections with combinations of anti-inflammatory, anti-bone resorptive agents, antioxidants, passive immunization in order to minimize the risk of sequalae[13]. Treatment modulating or inhibiting the inflammatory mediators may improve survival of patients with staphylococcal sepsis[14]. Studies in mice using corticosteroids in conjunction with antibiotics indicated a decreased morbidity and mortality[15]. It is also suggested to combine antibiotics with biological agents that have more selective effects such as anti-inflammatory cytokines[16]. Future therapies also envisage alternatives to direct microbial killing such as blocking disease progression by neutralizing specific

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virulence factors or boosting key innate immune system[17]. Protective and anti-inflammatory effects of dietary vitamin supplementation against *S. aureus* infection have been reported[18]. The B-vitamin, riboflavin may also play as an antioxidant in inflammatory reaction[19]. Therefore, riboflavin may be an effective therapeutic agent in septic arthritis[20]. Early administration of antibiotics eradicates the bacteria but does not stop joint destruction[21]. However, ampicillin was found to be effective against more than 70% of coagulase positive and coagulase negative staphylococci isolated from horses with osteomyelitis[22]. Efficacy of treatment with ampicillin alone or in combination with riboflavin on the *S. aureus* infection induced arthritis has been reported in this study.

Materials and Methods

Materials—Ampicillin, gentamicin, streptomycin, erythromycin were obtained from Hi Media Laboratories, Bombay, while chloramphenicol was from Sigma Chemicals, USA. Riboflavin and reagents for the anti-oxidant enzyme assay were purchased (from E. Merck, Bombay). ELISA kits for determining mouse cytokine levels (purchased from Bender Med Systems, Viena, Austria). All other chemicals used were of analytical grade.

Animals—Male Swiss albino mice, 6–8 weeks of age with body weight 20 ± 4 g 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*. Animals were maintained at 21–24 °C and 40–60% humidity with a 12-h light dark cycle and were fed with normal rodent diet.

Preparation of bacteria and culture conditions—S. aureus isolate (SCRL-28) used in this study was obtained from the blood of an adult male patient having septic arthritis at Scientific Clinical Research Laboratory, Calcutta, West Bengal, India. The isolate catalase positive and coagulase positive was maintained in our laboratory. Few clinical isolates of *S. aureus* were used extensively in a mouse model of arthritis with short term but non-lethal infection[23,24]. The *S. aureus* strain (SCRL-28) used in this study was also isolated from synovial tissue homogenate of Swiss albino mice after single i.v injection. Bacteria recovered from blood and spleen are found to be definitely *S. aureus*, since this specific mannitol agar culture media differentiates *S. aureus* from other catalase-positive and gram-positive cocci like *Staphylococcus epidermidis*. Moreover, after the antibiotic sensitivity assay of the strain tested for 3-5 antibiotics it was found to be sensitive to chloramphenicol (30 µg/disc), gentamicin (4 µg/disc) and resistant to erythromycin (15 µg/disc) and streptomycin (10 µg/disc) when performed *in vitro* disc agar diffusion. Effects of ampicillin in *S. aureus* induced arthritis were not reported, for that ampicillin was chosen as the possible protective antibiotic against TSST-1 and coagulase positive staphylococci. *S. aureus* strain grown overnight in Luria Bertini broth were diluted with fresh broth and cultured until mid-logarithmic phase of growth. Bacteria were harvested, washed twice with sterile saline and adjusted to the desired inoculum spectrophotometrically before injection (OD_{620} =1.6 for 1.0×10^9 CFU/ml for *S. aureus*) and the CFU were confirmed by serial dilution and culture on blood agar.

Mouse infection to determine optimum inoculum size of S. aureus for induction of arthritis—To determine effective dose of viable *S. aureus* cells for induction of septic arthritis, mice (5 mice for each dose) were infected i.v with graded doses of *S. aureus* (SCRL-28) in 0.2 mL of physiological saline through the tail vein. Control mice were injected with 0.2 mL of sterile physiological saline. Mice were sacrificed at 15 day post infection.

Treatment of S. aureus infected mice with antibiotic (ampicillin) followed by vitamin B2 (riboflavin)—Starting on day 0 after injection of *S. aureus* (SCRL-28) (5×10^6 cells/mL), ampicillin, dissolved in sterile PBS, was injected (i.p) into mice (6 mice per experimental group) at a single dose of 100 mg/kg after 24 h of infection[25]. Then fresh solution of riboflavin was prepared on the day of antibiotic treatment and 160-200 µL of riboflavin corresponding to 20 mg/kg body wt in mouse (approx 19 mg of vitamin B2) was given ip to the same mice after 2 h of antibiotic treatment[20]. 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—Blood (0.5 mL) was obtained on days 3, 9, and 15 after *S. aureus* infection by retro-orbital sinus bleeding before killing at selected intervals. Blood from each infected mice was plated on mannitol salt agar selective media. Then mice were killed, and spleen and synovial tissue from the joint were collected. Spleen was homogenized in 0.5 mL of

sterile PBS; 100 µL of this homogenate was spread on mannitol agar plate and incubated for 48 h at 37 °C. Viable bacterial colonies were then counted to evaluate the numbers of bacterial CFU in each homogenate. To avoid false positive results due to contamination, an isolate was considered positive when 15 or more *S. aureus* colonies were present. Results were expressed as number of bacterial CFU/mL of blood and per gram of spleen or synovial tissue[26]. Bacteria recovered from blood or spleen were definitely *S. aureus*, since 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. *S. aureus* also can ferment mannitol into acid detected hereby the change in *p*H indicator from red to yellow. Number of bacterial CFU obtained from either blood or spleen were not false positive, since bacterial presence was defined as 15 CFU or more for blood or tissue which are even higher in this study[3]. Bacterial colonies were tested for catalase and coagulase positivity.

Experimental evaluation of arthritis—Briefly, SCRL-28, 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 6 to 8 wk received either 5×10^6 CFU *S. aureus* in 100 µL PBS injected i.v. via the tail vein, or 100 µL PBS alone. Individual mice were observed daily for up to 15 days, blind to genotype or 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 caliper graduated 0.1 cm increments by carefully measuring the width and thickness of each wrist and ankle joints. The daily mean value of diameter of all the wrists or ankles for each group /Total number of wrists or ankles measured in each group. This average value represented the severity of wrist and ankle joints swelling[27].

Sample preparation for cytokine measurement—Blood samples from mice infected with 5×10^6cells/mL of *S. aureus* and from uninfected, untreated control mice were obtained by cardiac puncture under ether anesthesia at selected intervals. The Blood (0.2 mL) was transferred to micro-centrifuge tubes and allowed to clot at 4 °C. These tubes were then centrifuged at 10,000 rpm for 5 min at 4 °C. Supernatant pale yellow colored serum was pipette out carefully with the help of micropipettes into fresh micro-centrifuge tubes, labeled and stored at −80 °C for cytokine analysis. In each experiment, the mice were coded to ensure that the observer was blinded. Synovial tissue homogenate and serum from different groups were normalized to the protein content by Bradford method before the assay and levels of cytokines were determined by ELISA according to the manufacturer's instruction in a BioRad ELISA Reader.

Articular neutrophil accumulation—Myeloperoxidase (MPO) enzyme activity was analyzed as index of neutrophil infiltration in the synovial tissue, because it is closely related with 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 m*M* Tris–HCl, (*p*H 7.0), EDTA, sucrose and protease inhibitor cocktail and then centrifuged at 2,000g for 10 min at 4 °C. The supernatants were sterilized by passing through a Milipore filter (0.45 µm pore size) and stored at − 80 °C until analysis. Protein levels in the tissue homogenates were determined by Bradford method. An aliquot of the supernatant was allowed to react with a solution of O-dianisidine dihydrochloride (0.167 mg/mL) and 0.005% H_2O_2. 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[24].

Estimation of serum glutamate oxalate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) activity—Non-hemolyzed serum(100 µl) was mixed with 0.5 mL of glutamic oxaloacetic transaminase substrate and incubated for 1 h at 37 °C. Then 0.5 mL of 2, 4-dinitrophenyl hydrazine solution was added and kept for 15 min at room temperature. Then, 5 mL of 0.4N NaOH was added and mixed and kept at room temperature for 20 min. Intensity of the developed colour was read at

540 nm after setting the instrument at zero density with water. Decrease in density represents decrease in a-ketoglutarate from which the activity was calculated[28].

Preparation of tissue homogenates—Weighed amounts of liver, kidney, spleen and hearts were homogenized (10% w/v) in ice-cold 50 m*M* potassium phosphate buffer (*p*H 7.8), with the help of a power driven Polytron homogenizer. The homogenate was then centrifuged at 8,000g for 15 min at 4 °C. Supernatant was decanted off and stored at −20 °C until further analysis.

Estimation of tissue protein—Protein content of tissue homogenates, supernatant and serum was estimated by dye binding technique according to Bradford. It is a colorimetric assay that uses Coomassie brilliant blue (G-250). When this reagent binds to proteins at an acidic *p*H, there is an absorbance shift, and the color change was read spectrophotometrically at 595 nm.

Measurement of lipid peroxidation level (LPO)—Hepatic tissue was homogenized (10%) in ice-cold 0.9% saline (*p*H 7.0) with a Potter Elvejhem all glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30 s and the levels of the lipid peroxidation products in the homogenate was determined as Thio-barbituric acid reactive substances (TBARS) and the values were expressed as nmoles of TBARS per mg protein[29].

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. Values were expressed as nmoles of GSH per mg protein[30].

Measurement of activity of antioxidant enzymes—Superoxide dismutase (Cu-Zn SOD) activity was measured by hematoxylin auto oxidation method of Martin *et al*[31].

Catalase was assayed by measuring the breakdown of hydrogen peroxide (H_2O_2) according to the method of Beers and Sizer. The enzyme activity was expressed as micromoles of H_2O_2 consumed per min per mg protein[32].

Concentration of ampicillin in mouse sera following a single 100 mg/kg i.p dose—During separate *in vivo* investigations, 0.5 mL of intracardiac blood was obtained at different time following i.p. administration of ampicillin (i.e. day 3, 9, 15 post administration). Serum antibiotic concentrations were determined by agar diffusion bioassay technique. Dilutions of antibiotic containing sera, antibiotic standard of known conc. were made with antibiotic free mouse sera and sterile 6 mm diameter paper disks were used for all determinations. Plates were incubated for 24 h at 37 ^{o}C and the concentrations were determined by plotting of the zone sizes against standards of known concentration[25].

DNA isolation from S. aureus recovered from synovial tissue—Bacterial culture was grown in brain heart infusion broth prior to extraction of total DNA. Total DNA was isolated from 2.5 mL of brain heart infusion broth culture grown overnight for all the bacterial strains used in the study as described previously[33]. DNA samples were dissolved in Tris-EDTA buffer (10 m*M* Tris chloride, 1 m*M* EDTA [*p*H 8.0]), and the concentration was determined as micrograms per milliliter according to A_{260} values. Template DNA in amounts ranging from 10 to 1,000 ng was used in the study.

PCR (polymerase chain reaction) amplification and agarose gel electrophoresis—Primers for PCR were synthesized by Osmium Biosolutions on sequence published by Becker et al, 1998 for TSST gene. The sequence for TSST gene primer was $TSST_1$ 5'-AAGCCCTTTGTTGCTTGCG-3' and $TSST_2$ 5'-ATCGAACTTTGGCCCATACTTT-3'. The PCR amplifications were performed in a volume of 25 µL containing 20 -90 ng/µL DNA, 1 X PCR buffer, 3 m*M* $MgCl_2$, 200 µ*M* dNTPs, 20 pmols primers and 1.25 IU TAQ polymerase. An initial cycle of 96 ^{o}C for 5 min was followed by 35 cycles of 94 ^{o}C, 2 min at 54 ^{o}C and 1 min at 72 ^{o}C. Final extension was performed at 72 ^{o}C for 7 min. The tubes were placed in a BioRad, MJ-Mini thermocycler. PCR products were visualized on a 2% agarose gel stained with ethidium bromide and the size of the product was estimated using 100 bp DNA ladder[34].

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 <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 done as post hoc test for multiple comparisons of means of different groups when significant F value was found[35].

Results

With the increase in inoculum size of *S. aureus* (SCRL-28) there was gradual increase in the bacterial

density both in spleen and joint synovium correlating with survivality and induction of arthritis in mice when the *S. aureus* inoculum size was at 5×10^6 cells/mL (Table 1). In isolates obtained 3 days after start of infection, growth of *S. aureus* was noted not only in joints but also in blood and spleen. In contrast, in the isolates obtained 15 days after inoculation, growth of bacteria was still prominent in the spleen and joint as compared to blood. However, treatment of mice with ampicillin after infection followed by riboflavin completely eradicated the bacteria from blood and also significantly reduced bacterial burden both in spleen and synovial tissue (P<0.05) (Table 2). Result showed that there was significantly increased swelling of knee and elbow joints in pathogenic strain *S. aureus* (SCRL-28) infected mice after 3, 9 and 15 days of infection compared to uninfected control group (P<0.05). Treatment of mice with ampicillin alone or in combination with riboflavin after *S. aureus* infection showed significant gross reduction in arthritis as compared to *S. aureus* induced arthritis at 15 dpi (Table 3).

Serum TNF-α (Fig. 1a) and IFN-γ (Fig. 1c) levels but not IL-6 (Fig. 1b) were increased significantly after infection (P<0.05). Treatment of mice with Amp alone or in combination with riboflavin after infection found to be significantly down regulate the serum TNF- α at day 3 and day 9. However, Amp alone or in combination with Ribo could also decrease the serum IFN-γ significantly at day 3 and 15. In contrast, administration of either ampicillin alone or in combination with riboflavin after *S. aureus* infection showed reduced TNF-α content in the synovial tissue (Fig. 1e) and elevated IL-10 (Fig. 1h) release at 9 and 15 days post infection as compared to *S. aureus* alone.

Activity of MPO enzyme was significantly higher only for the pathogenic strain, SCRL-28 than the vehicle group. When ampicillin was administered alone or in combination with riboflavin it caused significant (P<0.05) reduction of tissue MPO enzyme activity at day 3 and day 9 post infection (Fig. 2).

Agarose gel electrophoresis picture clearly showed the presence of TSST-1 gene in the bacteria recovered from the synovial tissue of *S. aureus* (SCRL 28) infected mice (Fig. 3). However, treatment of

Table 1—Effects of *S .aureus* inoculum size on arthritis, mortality and bacterial burden in Swiss albino mice. Results were reproduced in 3 repeated experiments
[Values are mean ± SD of 5 mice per group]

Inoculum size (cells/mL)	No. of mice dead	No. of arthritic mice	Bacterial burden (mean ± SD) at 15th day post infection		
			Blood	Spleen	Synovial tissue
Uninfected control	0	0	6 ± 1	10 ± 0.58	9 ± 0.6
[a]5×10^6	0	5	30 ±0.37	467 ± 37.5	520 ± 28.3
10^7	1	4	50 ±1.03	1867 ± 47	875 ± 88.5
2×10^7	2	3	80±14	2000 ±70.7	975 ± 17.7
4×10^7	2	3	120±14	2400 ±283	1225 ± 230
8×10^7	3	2	4720±50	6850 ±106	3500 ± 742

Table 2—Recovery of bacteria from blood, spleen and synovial tissue after single *in vivo* injection of *S. aureus* (SCRL-28) followed by ampicillin and riboflavin treatment after an interval of 2 h respectively
[Values are expressed as mean ± SD of 6 mice per group]

Days post infection	CFU/mL of blood				CFU/g of spleen tissue				CFU/g of synovial tissue			
	Control	*S.aureus*	*S. aureus* + Ampicillin	*S. aureus* + Ampicillin + Riboflavin	Control	*S* .aureus	*S. aureus* + Ampicillin	*S. aureus* + Ampicillin + Riboflavin	Control	*S. aureus*	*S. aureus* + Ampicillin	*S. aureus* + Ampicillin + Riboflavin
Day 3	0	888±17.7	73 ± 18.4	30 ±14.14	0	3348±67	735±120.2	254 ±112	0	3000±141	1700±283	1325±106
Day 9	0	200±14.1	145±7.07	80±28.28	0	3100±566	375±21.21	180±28	0	1980±28	1275±106	650±71
Day 15	0	84±8.49	20±0	10±0	0	1500±141	200±0	75±0	0	655±77.8	88±31	33±4.24

All the values are significant at 0.05 level in the population mean
a = uninfected control versus *S. aureus*; significant at P<0.001.

ampicillin in combination with riboflavin after infection showed reduced production of TSST- 1.

Effects of ampicilin-riboflavin co-therapy against *S. aureus*-infection induced oxidative stress in various tissues of mice and its relation with progression of septic arthritis

Cardiac tissue—Infection of mice with *S. aureus* did not exhibit any significant change in activity of SGOT except at day 15. However, SGOT activity in *S. aureus* plus ampicillin treated group was found to be reduced significantly in day 3 and day 15 with almost no change in day 9 (data not shown).

Following infection of mice with *S. aureus*, the level of LPO was found to be increased in days 3, 9 and 15 significantly when compared to controls (Fig. 4a). However, a significantly decreased LPO

Table 3—Gross arthritic score and reduction (in percentage) of arthritis after treatment with ampicillin or riboflavin at day 3, 9 and 15 post infection in Swiss albino mice
[Values are mean ± SD of 6 mice per group]

Group	Treatment	Dose	Gross arthritic score at day 3, 9 and 15 post infection			% reduction after treatment at day 15
			Day 3	Day 9	Day 15	
1	Uninfected control	-	0	0	0	0
2	*S .aureus* infection	5×10^6/mL	18.94 ± 0.0152	21.31 ± 0.3701	23.1 ± 0.1212	0
3	*S .aureus* + Ampicillin	Amp 100 mg/kg	19.17 ± 0.4085	20.46 ± 0.1817	19.95 ± 1.0275	[a]13.63
4	*S .aureus* + Ampicillin + Riboflavin	Amp 100 mg/kg + Ribo 20 mg/kg	18.78 ± 0.0764	16.76 ± 0.2650	15.11 ± 0.0378	[b]34.58

% reduction indicates the reduction of arthritic score at 15 dpi as compared with group 2 (*S. aureus* alone)
a = S. *aureus* alone versus *S. aureus* + Amp, significant reduction; $P<0.05$
b = S. *aureus* alone versus *S. aureus* + Amp + Ribo, significant reduction; $P<0.001$

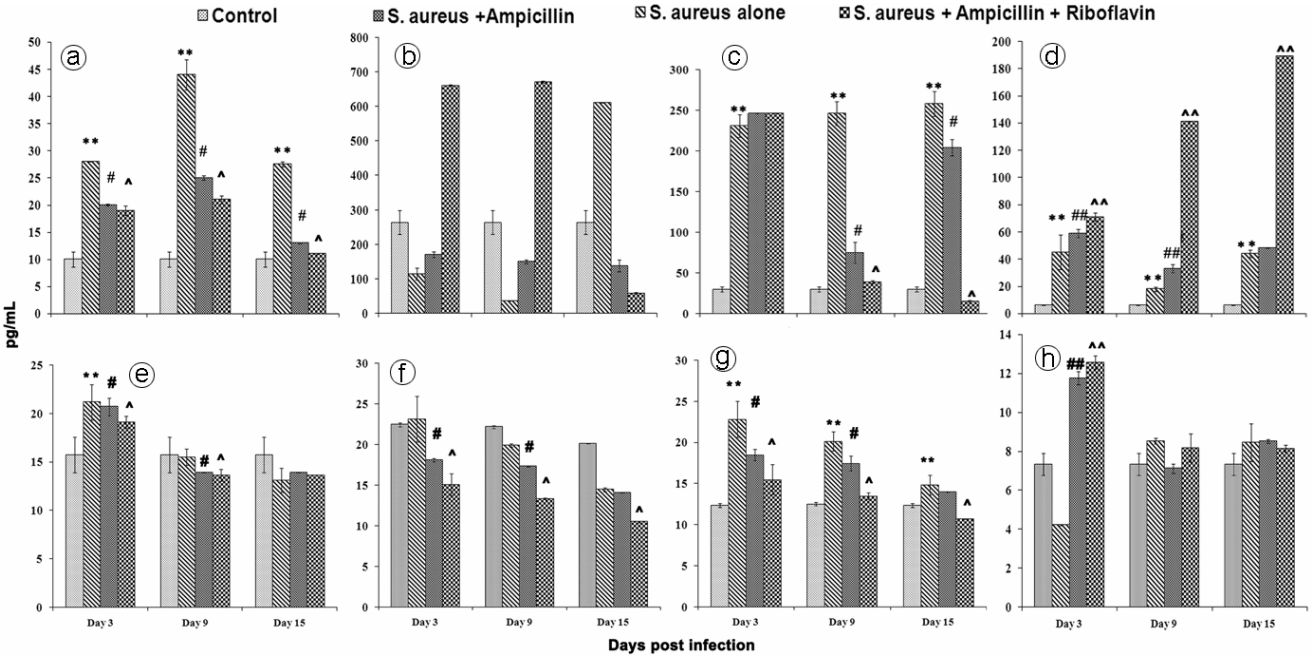

Fig. 1—Serum levels of (a)-TNF-∝; (b)-IL-6; (c)-IFN-ϒ and (d)-IL-10 and synovial joint levels of (e)-TNF-α; (f)-IL-6; (g)-IFN-γ and (h)-IL-10 in different groups of mice at 3, 9 and 15 days post infections [Values are expressed as Mean ± SD and are significant ($P<0.05$) from 6 mice in each group]. [**]without infection control versus *S. aureus* (SCRL-28) alone, significant increase; [##]*S. aureus* (SCRL-28) alone versus *S. aureus* (SCRL-28) + Amp, significant increase; [#]*S. aureus* (SCRL-28) alone versus *S. aureus* (SCRL-28) + Amp, significant decrease; [^^]*S. aureus* (SCRL-28) alone versus *S. aureus* (SCRL-28) + Amp + Ribo, significant increase; [^]*S. aureus* (SCRL-28) alone versus *S. aureus* (SCRL-28) + Amp + Ribo, significant decrease.

level was also observed in the mice treated with *S. aureus* plus ampicillin and riboflavin compared to mice infected with *S. aureus.*

The GSH level did not show any alteration at all the time-points tested when the mice were infected with *S. aureus*. However, in the *S. aureus* plus ampicillin treated group, the GSH levels increased significantly at day 3 post infection. A further increase in GSH level was noted in day 3 and day 15 in *S. aureus* plus ampicillin and riboflavin treated mice compared to the group treated with *S. aureus* plus ampicillin. The extent of increase in GSH level in *S. aureus* plus ampicilin and riboflavin treated mice was much higher in day 3 in comparison to days 9 and 15 (Fig. 4b).

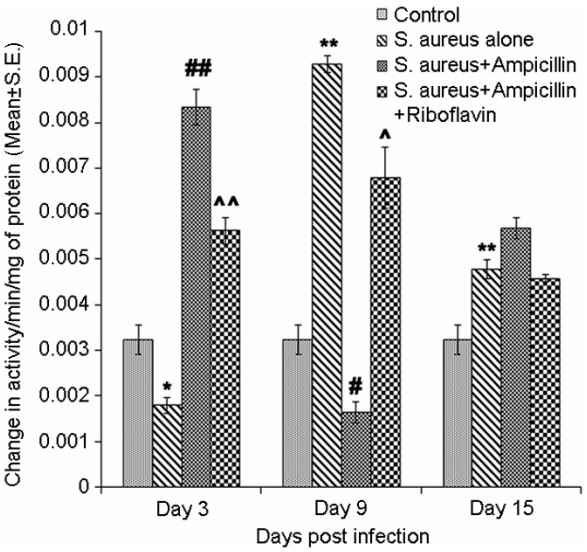

Fig. 2—Articular neutrophil accumulation determined by estimation of myeloperoxidase (MPO) activity. [Values are expressed as Mean ± SD and are significant (P<0.05) from 6 mice in each group]. As in Fig. 1; *without infection control versus *S. aureus* (SCRL-28) alone, significant decrease.

A significant decrease in the activity of SOD was observed in mice treated with *S. aureus* in days 3 and 9 but not in day 15. The SOD activity was also found to be significantly increased in day 3 in mice treated with *S. aureus* plus ampicillin and when compared to activity in day 3 of mice treated with *S. aureus*. Moreover, the SOD activity was found to be significantly increased in days 3 and 9 in *S. aureus* plus ampicillin and riboflavin treated mice compared to *S. aureus* treated mice (Fig. 4c).

Catalase activity decreased significantly in days 3 and 9 in mice infected with *S. aureus* compared to controls. The results show that the catalase activity of *S. aureus* plus ampicillin group did not differ significantly from the mice treated with *S. aureus* alone. However, catalase activity increased

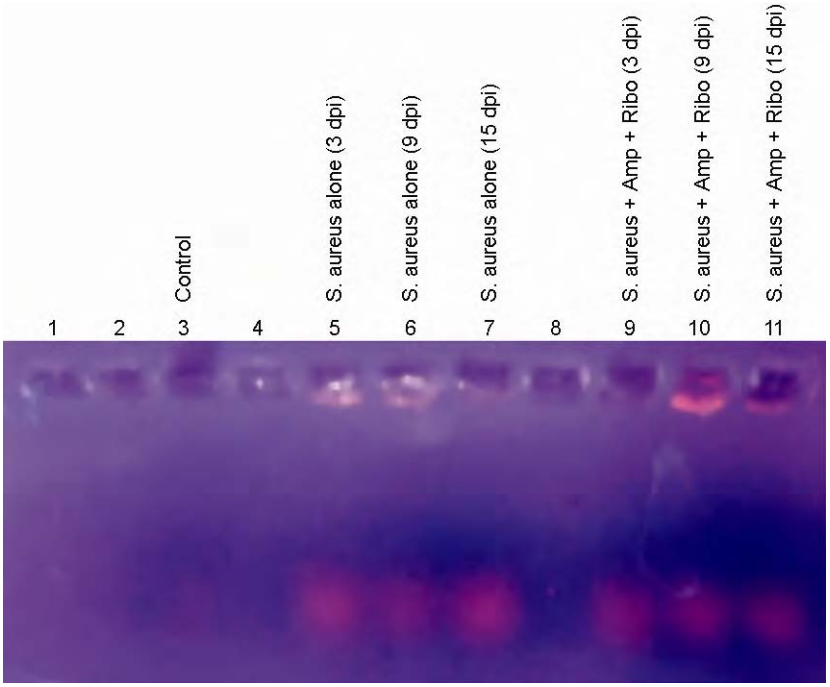

Fig. 3—Presence of TSST-1 gene in *S. aureus* (SCRL-28) recovered from synovial tissue of infected mice-PCR products of DNA isolated from *S. aureus* (SCRL-28) recovered from synovial tissue of infected group were transferred in agarose gel to show the presence of TSST-1 in separate lanes.

significantly in all the time-points tested in the *S. aureus* plus ampicillin and riboflavin group when compared to *S. aureus* plus ampicillin treated mice (Fig. 4d).

Hepatic tissue—Activity of SGPT in *S. aureus* treated mice did not differ significantly compared to control (data not shown). Level of LPO (Fig 4e) increased significantly in day 3 and day 9 in *S. aureus* treated mice compared to control but LPO level did not increase in day 15 compared to respective control. The LPO level decreased significantly at day 3 and day 9 in *S. aureus* plus ampicillin compared to *S. aureus* treated mice. The LPO level, however, was found to be significantly lower at day 3 and 9 in *S. aureus* plus ampicillin and riboflavin treated mice compared to *S. aureus* treated mice.

Level of GSH (Fig. 4f) in the *S. aureus* treated mice did not differ significantly at day 3 but differ significantly at day 9 compared to controls. Similarly, the GSH level increased significantly in *S. aureus* plus ampicillin treated mice at all the time-points tested when compared to *S. aureus* only treated mice. It is interesting to note that when the mice were treated with *S. aureus* plus ampicilin and riboflavin, there was a several fold increase in GSH level which remained increased significantly also at day 9 and day 15 compared to *S. aureus* plus ampicillin treated mice. SOD activity increased significantly at day 3 while decreased significantly at day 9 and 15 in *S. aureus* treated mice compared to respective controls. The SOD activity increased further at day 9 and day 15 in *S. aureus* plus ampicillin treated mice when compared to *S. aureus* only treated mice. However, SOD (Fig. 4g) activity was found to decrease significantly in *S. aureus* plus ampicillin and riboflavin treated mice compared to mice treated with *S. aureus*.

A significant decrease in catalase activity was observed at all the time-points tested in *S. aureus* treated mice compared to respective controls. However, the catalase activity was significantly different at day 15 in *S. aureus* plus ampicillin treated mice compared to *S. aureus* treated mice whereas at other time-points the changes in the activity of this enzyme was found statistically not significant. The catalase activity was found to be significantly higher in days 3, 9 and 15 in *S. aureus* plus ampicillin and riboflavin treated mice compared to *S. aureus* plus ampicillin treated mice (Fig. 4h).

Splenic tissue—Significantly increased level of LPO (Fig. 5a) at day 3 and day 9 and a significantly decreased level of LPO at day 15 in mice with *S. aureus* infection were observed compared to the respective controls. A significantly increased level of LPO was observed at day 3 and significant decreases at days 9 and 15 in mice treated with *S. aureus* plus ampicillin compared to *S. aureus* treated mice. However, when mice infected with *S. aureus* plus

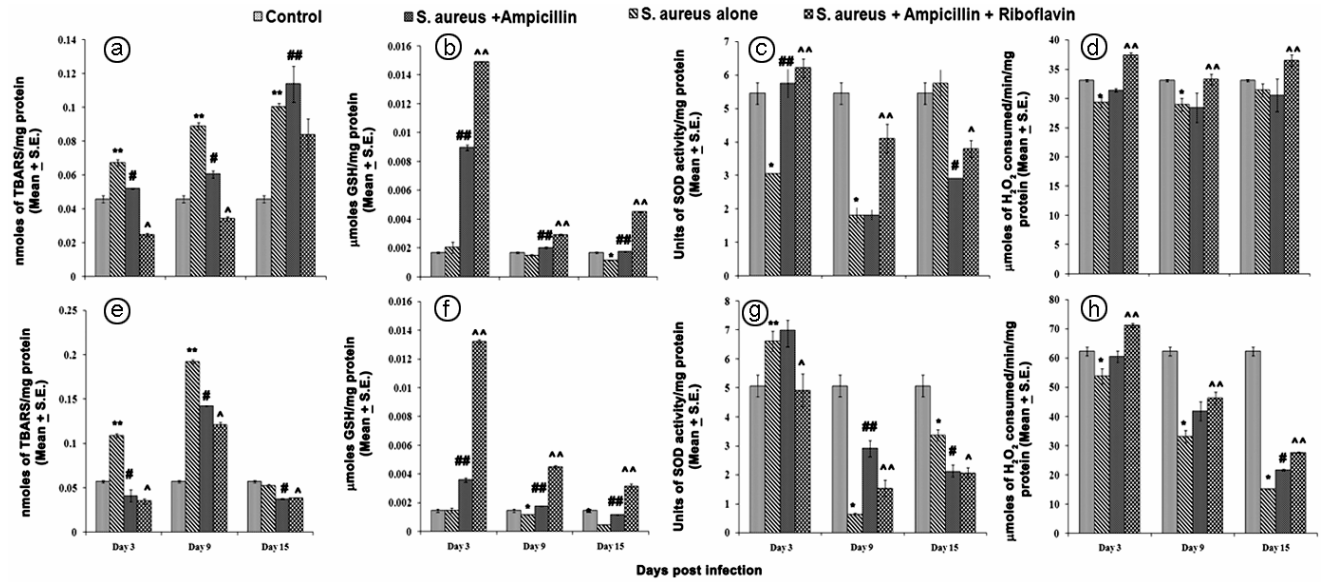

Fig. 4—Alteration in reduced glutathione level and SOD and catalase activity of cardiac and hepatic tissue respectively: (a)-cardiac LPO, (b)-cardiac GSH, (c)-cardiac SOD, (d)-cardiac catalase, (e)-hepatic LPO, (f)-hepatic GSH, (g)-hepatic SOD and (h)-hepatic catalase. [Values are expressed as Mean ± SD and are significant (P<0.05) from 6 mice in each group]. As in Fig. 1; *without infection control versus *S. aureus* (SCRL-28) alone, significant decrease.

treated with ampicillin and riboflavin, the level of LPO was also found to be significantly lower when compared to the *S. aureus* plus ampicillin treated mice. GSH level of splenic (Fig. 5b) tissue significantly increased at days 9 and 15 following infection with *S. aureus* compared to control mice but at day 3 there occurred almost no change compared to respective control. However, splenic GSH level of mice treated with *S. aureus* and ampicillin increased significantly at day 3 and decreased significantly at days 9 and 15 when compared to the group of mice treated with *S. aureus* only. The GSH level significantly decreased at days 3, 9 and 15 in the mice treated with *S. aureus* plus ampicillin and riboflavin when compared to the mice treated with *S. aureus* plus ampicillin.

SOD activity (Fig. 5c) decreased in spleen tissue after *S. aureus* infection compared to mice in control group. However, the activity change was found to be significantly decreased at day 3. Activity of SOD was also found to be significantly increased at days 9 and 15 in *S. aureus* plus ampicillin treated mice compared to *S. aureus* treated mice. Here again, the SOD activity at day 3 did not differ from the activity observed in the *S. aureus* treated mice. The SOD activity in the *S. aureus* plus ampicillin and riboflavin treated mice increased significantly at days 3, 9 and 15 compared to *S. aureus* treated mice. It is interesting to note, however, that at day 15 the SOD activity was found to be more than double in the *S. aureus* plus ampicillin and riboflavin treated mice compared to *S. aureus* plus ampicillin treated mice.

Significant decrease of splenic catalase activity (Fig. 5d) at days 9 and 15 and a non-significant decrease at day 3 in the mice treated with *S. aureus* compared to the respective controls. However, the activity of this antioxidant enzyme was found to be increased significantly at days 9 and 15 with an insignificant change at day 3 in mice treated with *S. aureus* plus ampicillin compared to the group of mice treated with *S. aureus* only. Interestingly, in this study a significantly increased catalase activity in splenic tissue at days 3, 9, and 15 in *S. aureus* plus ampicillin and riboflavin treated mice was observed compared to *S. aureus* plus ampicillin treated mice. Furthermore, a more than double increment in the activity of catalase is also evident at day 15 in the *S. aureus* plus ampicillin and riboflavin treated mice compared to *S. aureus* plus ampicillin treated mice of the same time-point.

Renal tissue—More than two fold in increase in the level of LPO of the renal tissue (Fig. 5e) at days 3, 9, and 15 in the mice treated with *S. aureus* compared to the control mice. However, the level of LPO decreased significantly in days 3, 9 and 15 in *S. aureus* plus ampicillin treated mice compared to the mice treated with *S. aureus* only. But a further significant decrease in the LPO level was evident at days 3, 9 and 15 in the *S. aureus* plus ampicillin and riboflavin treated mice when compared to mice treated with *S. aureus* plus ampicillin. This indicates that antibiotic-antioxidant co-therapy is capable of reducing the level of LPO in the kidney tissue.

Significant increase in GSH level of the renal tissue (Fig. 5f) at days 9 and 15 in mice treated with *S. aureus* compared to control but there occurred no change at day 3 when compared to the respective controls. Likewise, a significant decrease (more than two fold) in the kidney tissue GSH was observed at days 9 and 15 in mice treated with *S. aureus* plus ampicillin and compared to the mice treated with *S. aureus* only. Here also at day 3 the level of GSH did not show any statistically significant change when compared to the levels observed in mice treated with *S. aureus* only. The figure further reveals that at day 3, the level of this antioxidant increased significantly (more than two fold change) in mice treated with *S. aureus* plus ampicillin and riboflavin compared to mice treated with *S. aureus* plus ampicillin indicating again a protective role of antibiotic-antioxidant co-therapy against *S. aureus* –induced oxidative stress in mice in experimental infection.

A significant decrease in the activity of renal SOD (Fig. 5g) was observed at days 3, 9 and 15 in mice treated with *S. aureus* compared to respective controls. Activity of kidney SOD in the mice treated with *S. aureus* plus ampicillin was found not to be statistically significant at days 3, 9 and 15 when compared to the activity observed in the kidney tissues of *S. aureus* treated mice. However, when the mice were treated with *S. aureus* plus ampicillin and riboflavin, the SOD activity of kidney tissue significantly increased at days 3, 9 and 15 and the increase at day 15 was found to be more than double when compared to the activity observed in *S. aureus* plus ampicillin treated mice. Results indicate the efficacy of the antibiotic-antioxidant co-therapy in abating the changes due to oxidative stress following *S. aureus* infection of the mice.

Catalase activity of the renal tissue (Fig. 5h) was decreased significantly at days 3, 9 and 15 (more than double) following infection of the mice with *S. aureus*

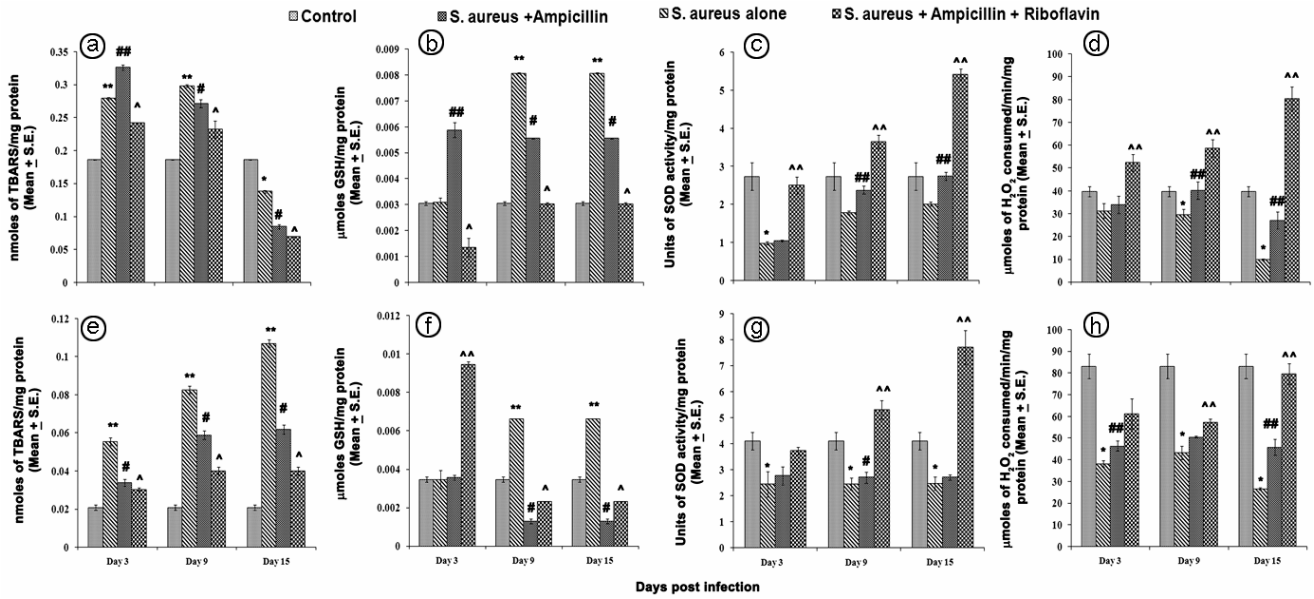

Fig. 5—Alteration in reduced glutathione level and SOD and catalase activity in splenic and renal tissue respectively: (a)- splenic LPO, (b)- splenic GSH, (c)- splenic SOD, (d)-splenic catalase, (e)-renal LPO, (f)- renal GSH, (g)- renal SOD and (h)- renal catalase. [Values are expressed as Mean ± SD and are significant (P<0.05) from 6 mice in each group]. As in Fig. 1; *without infection control versus *S. aureus* (SCRL-28) alone, significant decrease.

and compared to the respective controls. However, there occurred a significant increase in the activity of catalase of the kidney tissue at days 3 and day 15 in the mice treated with *S. aureus* plus ampicillin compare to mice treated with *S. aureus*. The activity of the enzyme was found to be significantly higher at days 3, 9 and 15 in *S. aureus* plus ampicillin and riboflavin treated mice when compared to the activity observed in *S. aureus* plus ampicillin treated mice. Furthermore, it is interesting to note that activity of catalase in *S. aureus* plus ampicillin and riboflavin treated mice was found to be near control value indicating again a protective effect of antibiotic-antioxidant co-therapy against oxidative stress induced by infection of the mice with *S. aureus*.

Discussion

In this study the antibiotic and the vitamin treated groups of mice were protected from arthritis and the corresponding inflammatory changes and productions of ROS were found to be reduced. Circulating staphylococci disseminate to virtually all tissues but less than a week they are cleared from the blood with great exceptions in the spleen and joints. In a separate *in vitro* experiment it was found that MIC of Amp for SCRL-28 was 62.5 µg/mL. Therefore, 100 mg/kg dose was selected in order to have antibiotic levels detected by bioassay for longer periods of time. It was reported that plasma concentrations of ampicillin were consistently higher at 2 h interval after s.c administration. Therefore in this study riboflavin was administered just after 2 h of antibiotic treatment. In these experiments performed, the final result is that the staphylococci were cleared faster from the blood and spleen supporting the combined use of riboflavin and ampicillin in the prevention of infection produced by *S. aureus* (SCRL-28). After 3 days post infection (dpi), bacterial burden was cleared from blood but increased in spleen. It might indicate that with the progression of bacterial arthritis, the bacterial load increases in the specific tissues.

Mice inoculated with TSST-1 positive SCRL-28 displayed significantly higher levels of TNF-α and IFN-γ than did those control. Levels of IL-6 only in the synovial tissue of mice with *S. aureus* induced arthritis are highly increased; however, treatment of mice with ampicillin and riboflavin caused decreased synovial IL-6 content at day 9 and 15. Since levels of IL-6 are highly correlated with disease severity[36]. Our study demonstrated that Amp + Ribo are critical for anti-inflammatory and joint protection treatment of staphylococcal arthritis. Since IL-6 and IFN-γ are known to be potent B-cell activators and B lymphocytes contribute to the pathogenesis of arthritis, possibly by more efficient presentation of auto antigens, these effects are also inhibited as

endogenous IL-6 and IFN-γ levels are reduced by Amp + Ribo treatment.

Administration with ampicillin followed by riboflavin after *S. aureus* infection resulted in an increase of IL-10 production, which is the most potent anti-inflammatory cytokine and is a potent inhibitor of TH1 cytokines including IFN-γ. Moreover IL-10 is essential for elimination of bacteria and therapy for protection against septic arthritis[37]. It was also demonstrated that TH2 response become dominant in *S. aureus* infection and that IL-10 play a protective role by inhibiting IFN-γ[38]. The conclusion is that ampicillin and riboflavin treatment leads to reduction of live *S. aureus*, resulted in a decrease in exotoxin production, which in turn stimulated a reduced production of the proinflammatory cytokines including TNF-α and IL-6. Therefore, blockage of IL-6 secretion *in vivo* might be developed as a therapeutic application against *S. aureus* arthritis.

As recruited or infiltrated polymorphonulear neutrophils (PMN) at the synovial tissue at day 3, 9 or 15 post-infection are directly activated by bacterial constituents, these cells may be seen as part of the innate immune system actively involved in the development of arthritis. The role of PMN migration to the infectious focus may be responsible for the severity and outcome of arthritis and tissue destruction.

It is clear that during infectious disease or inflammatory processes, there is an alteration in capacity to neutralize ROS and to activate antioxidant enzymes and deactivate endogenous free radicals. The present results reveal that treatment of mice with *S. aureus* induces oxidative stress in several of mouse organs like liver, heart, spleen and kidney. This is reflected in an elevated level of lipid peroxidation and a decreased level of reduced glutathione, the primary biomarkers of oxidative stress. The involvement of oxidative stress in *S. aureus* infection in mice is also evident from the altered activities of superoxide dismutase and catalase activities of the liver, heart, spleen and the kidney tissues. Changes in myelo-peroxidase activity are also strongly indicative of oxidative stress. These changes observed post-infection are indicative of the generation of oxygen based reactive species in the respective tissues. However, when the mice were co-treated with ampicilin, the level of oxidative stress was reduced to a significant degree. This may be due to either killing of bacteria by the antibiotic and reducing thereby the stress burden. Whether the molecular structure of ampicillin has the potential of acting as an electron sink, apart from its antibacterial capacity remains to be investigated.

Earlier studies further reveal that when the mice were co-treated with riboflavin, a known antioxidant[39] and ampicillin, the level of oxidative stress, in almost all the tissues studied, was found to be decreased. Activities of the antioxidant enzymes were found to be almost near normal in the tissues studied. Results indicate strong antioxidant intervention against *S. aureus* infection when the riboflavin and the antibiotic were co-administered. Potent antioxidant activity of the combination may be due to a synergistic action. Antioxidant potential of riboflavin has been the subject of much current research[40] and this may not need repetitive doses. The importance of riboflavin as an antioxidant may also lie in its radical scavenging potential due its ability to get converted enzymatically within the tissues to flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), the two important flavin co-enzymes which has tremendous bearing on the oxidative metabolism of the tissues. The generation of oxidative stress may also be due to the depletion of these two flavin co-enzymes following infection of the mice with *S. aureus*. However, suitability of riboflavin as an antioxidant in long term infective condition in repetitive doses remains to be ascertained and appears to be an interesting area of research in intervention in chronically infective situation with antioxidant or combinations of antioxidant(s) and antibiotic. Although we emphasize the importance of further studies on this subject, we suggest the use of this type of co-supplementation for the general population since prevention is a much more effective approach than treatment of the disease.

Ampicillin concomitant with riboflavin treatment may regulate the *S. aureus* infection induced activation of inflammatory mediators and free radicals implicating a protective role of combined anti-oxidant and antibiotic co-treatment in septic arthritis.

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