

COMBINATION TREATMENTS USING VANCOMYCIN WITH IMMUNOMODULATORS TO MODULATE *STAPHYLOCOCCAL* ARTHRITIS

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ABSTRACT

Objective: *Staphylococcus aureus* is an intensely studied organism to cause septic arthritis. This study aims at using vancomycin to mitigate the bacterial burden in mice infected with pathogenic strain of *S. aureus* which is combined with the *in vivo* inhibition of nitric oxide (NO) and prostaglandin (PG) levels via aminoguanidine (AMG) and meclofenamic acid (MFA), respectively, to modulate the inflammatory conditions in bacterial arthritis.

Methods: Synergy between the drugs was performed via Checkerboard. The clinical signs of septic arthritis were recorded on the 3rd, 9th, and 15th days post-infection (DPI) including induction of arthritis, measuring bacterial density in blood, spleen and synovial tissue, blood parameters and cytokines (tumor necrosis factor alpha (TNF α), interferon gamma (IFN γ), interleukin-6 [IL-6], IL-10) levels, myeloperoxidase (MPO) and lysozyme activities, and histopathological examinations of the synovial joints.

Results: *S. aureus* infection showed a significant increase in bacterial densities and inflammation. AMG/MFA treatment alone showed no bacterial clearance since endogenous NO or PG had been limited for bacterial killing but observably down-regulated inflammatory upshots. Vancomycin co-treatment with AMG or MFA showed maximum mitigation of bacterial load as well as the inflammatory conditions, articular repair and decreased in the percentage of induction of arthritis.

Conclusion: Diminution of *S. aureus* burden in tissues via vancomycin and suppression of inflammatory by AMG or MFA may have shown a visibly potent therapeutic remedy to combat septic arthritis.

Keywords: Aminoguanidine, Meclofenamic acid, Septic arthritis, *Staphylococcus aureus*, Vancomycin

INTRODUCTION

Septic arthritis deputizes an agonizing ailment of joint infection either by direct invasion of microorganism inoculum into the joint, by conterminous spread from infected articular tissue or by hematogenous spread [1]. A majority of bacterial arthritis is caused by *Staphylococcus aureus* which may be ascribed to its wealth of pathogenic determinants: An ample variety of surface protein for colonization of host tissue, invasions to aid *S. aureus* spread in tissues (leukocidin, kinases, and hyaluronidase), surface factors to impede phagocytic engulfment (capsule protein A), the metabolic factors promoting their survival in the phagocytes (carotenoids), factors to camouflage immune system (protein A, coagulase), toxins to destroy host membranes (hemolysins and leukotoxins), exotoxins to nourish inflammation and damage host tissues (*Staphylococcal* enterotoxin-G, toxic shock syndrome toxins or TSST) and acquired components to resist antimicrobial interference in their growth, survival, and pathogenicity [2]. By such multifarious virulent weapons, *S. aureus* easily gets homed into the joint space, resides in the basement free synovial tissue stimulating the immune system to proliferate T-lymphocytes, boost the infiltration of neutrophils, macrophages and immunologically potent cells which try to affray the bacterial pathogenicity by effective pro-inflammatory substances (such as cytokines, nitric oxide [NO], prostaglandins (PGs), and various enzymes). These insurrections of conflict between the virulence factors of *S. aureus* and the host immune system ultimately trigger inflammatory conditions in the host resulting in severe sepsis [3].

Multitudinous combination therapies are being investigated, and scores of such experiments are under exploration which could mitigate the incidence of nosocomial infections, impoverish bacterial burden as well as heal the inflammatory outbursts. These can be achieved by paying attention on sufficing antibiotic prophylaxis, pre-treatment, and specific intervention of acute infection [4]. *S. aureus* can be dealt with an array of

antibiotics specially those which can be treated via a β -lactam group of antibiotics such as penicillin, oxacillin, or methicillin, that is, methicillin-susceptible *S. aureus* (MSSA). However, the evolution of methicillin-resistant *S. aureus* (MRSA) demanded development of new agents and approaches to keep up with the continuous strive to minimize *S. aureus* burden in the host body and to nullify the inflammatory outcome [4,5]. Depending on the strategies of bacterial evasion mechanisms, therapy innovations using novel antibiotics combinations have been the trend of biochemical, microbial, and immunological realms of research for a few years. It has been shown that after being treated by methicillin or oxacillin, cefazolin followed by cefradine treatment were found to mitigate the MSSA as well as borderline susceptible *S. aureus* and finally vancomycin treatment reportedly could minimize the MRSA strains only showing ineffectiveness to vancomycin-resistant *S. aureus* [6]. Thus, the choice of antibiotic in case of MRSA strains, conceivably be vancomycin, which is a unique glycopeptides antibiotic that acts by inhibiting second stage of the peptidoglycan synthesis in bacteria (by binding to the two D-alanine residues on the end of the peptide chains and by preventing them from interacting with the cell-wall cross-linking enzyme), via alteration of the permeability of the cell membrane and selectively inhibiting RNA synthesis [7,8].

Besides eradicating the *S. aureus* colonies from the infected tissue, focus should also be laid on abating the inflammatory consequences brought about by the pro-inflammatory cytokines (such as tumor necrosis factor alpha [TNF- α], interferon gamma [IFN- γ], and interleukin-6 [IL-6]), and other key pro-inflammatory substances such as NO and PG which possess the potential to stimulate severe inflammatory reactions [9]. The role of both NO and PGs in allergy, innate immunity, and inflammation are being studied for years, but they still remain areas of interest whose regulation can lead to diverse effects. A critical role has been proposed for NO in several infectious diseases and the interest to modulate the synthesis of endogenous NO to treat several diseases,

is the growing evidence implicating NO in inflammation, arthritis, diabetes, vascular complications, and wound healing. It is produced by inducible NO synthase (iNOS) has been shown to be involved in various inflammatory processes in a complex manner [10]. Furthermore, several anti-inflammatory drugs work by the mechanism of inhibition of the cyclooxygenase pathway (mainly COX-2) that produces PGs and thromboxanes from arachidonic acid [11].

Thus, to mitigate the inflammatory conditions, aminoguanidine (AMG), an inhibitor of NO synthesis or meclofenamic acid (MFA), an inhibitor of PG production in combination with the antibiotic, vancomycin has been investigated in this study to observe the degree of modulation of infection in *S. aureus*-induced septic arthritis in mice.

METHODS

Animal maintenance and survival rate study

Male Swiss albino mice, 6-8 weeks old, average body weight 20 ± 4 g, were used for all experiments. Upon arrival, mice were randomized into plastic cages with filter bonnets and sawdust bedding, followed by a 1-week quarantine period. Mice were housed 6 per cage with food and water *ad libitum*. Animal rooms were maintained at 21-24°C and 40-60% humidity with a 12 hrs light-dark cycle. All experiments were approved by the Institutional Animal Ethical Committee (IAEC) (Proposal number: IAEC-III/Proposal/Ph.D-URF/BB-03/2012 dated July 24, 2012). Both control and treated mice were monitored daily. The number of mice survived at different days post-infection (DPI) was noted [12].

Bacteria

Pathogenic *S. aureus* strains (P-1490, P-1488, and P-1486) were of a clinical isolate from NRS Hospital at Kolkata. In each experiment, bacteria were cultured on nutrient agar for 24 hrs at 37°C, inoculated into tryptic soy broth, and incubated for another 15 hrs. The organisms were collected by centrifugation and washed three times with 0.85% saline. The concentration of washed cells was adjusted spectrophotometrically at 550 nm. The numbers of viable *S. aureus* cells were established by plating serial 10-fold dilutions of a bacterial solution in 0.01 M phosphate buffered saline (pH 7.4) on nutrient agar. Mice were injected intravenously, 10^6 colony-forming unit (CFU)/mouse of average body weight of 20 g via the tail vein in 0.1 ml saline to each of the mice, grouped for each strain of *S. aureus* separately. Control mice received 0.1 ml of sterile saline through the tail vein. Bacterial colonies were routinely counted [12].

Test for synergism and antagonism

Checkerboard assay was performed to analyze the mode of interaction of the antibiotic, vancomycin with the immunomodulators, AMG or MFA in killing *S. aureus* [13]. For each combination, synergy test was using 96-well microtiter plate containing two drugs in two-fold dilutions ($4 \times$ minimum inhibitory concentrations [MIC] to $1/32 \times$ MIC), and they have been dispensed in a checkerboard fashion. Σ fractional inhibitory concentrations (FICs) were calculated to classify the effect of the combination of the drugs as the following: Synergistic, for FIC indexes ≤ 0.5 ; no interaction, for FIC indexes $>0.5-4$; and antagonistic, for FIC indexes >4 .

Treatment with antibiotics and immunomodulatory agents

Mice were infected separately with all the three strains of *S. aureus* (P-1490, P-1488, and P-1486) to assess the bacterial clearance from blood, spleen, and synovial tissue at 3, 9, and 15 DPI as well as induction of arthritis using these three strains. In a separate experiment, mice were infected with assumably the most potent *S. aureus* strain (P-1490). This infection was followed by treatment with the antibiotic, vancomycin at a dose of 23 mg/kg of body weight of mice which is calculated by the formula [14].

$$\text{Dose} = (C_{\max} * V_d) / F$$

Where, C_{\max} = Highest concentration in plasma = $\text{MIC} * 2^{(\text{dose interval} / \text{half-life})}$, V_d = Volume distribution, F = Systemic availability.

This is followed by peritoneal administration of immunomodulatory agents, MFA (COX-2 inhibitor) (10 mg/kg body weight) [15] and AMG (iNOS inhibitor) (400 mg/kg of body weight) [16]. Then, mice were sacrificed at 3, 9 and 15 DPI.

Swelling of wrist and ankle joints, arthritic scoring

Arthritis scoring was done according to Mal *et al.* [12]. Swelling of wrist and ankle joints was to determine the level of the inflammatory response in mice challenged with *S. aureus*. Before the experimentation, the paws of randomly selected and age-matched mice were measured, and the baseline paw size of the mice was determined. After infection, the mice paw diameter, wrist, and ankle joints were measured every other day for 15 days with a dial-type vernier-calliper graduated 0.1 cm, and the daily mean values were noted. This average value was used to infer the severity of swelling due to arthritis [17]. Percentage induction in arthritis per group of treated animals was calculated by considering the average paw diameter of the control group to be zero.

Determination of numbers of viable *S. aureus* in blood, spleen, and synovial tissue

Blood from each infected mice was plated on mannitol salt agar selective media. Spleen and synovial tissue were excised, weighed, homogenized, diluted in sterile saline and also plated on mannitol agar. Results were expressed as the number of bacterial CFU/ml of blood and per 100 mg of tissue [12].

Blood parameters of arthritis and inflammation

Serum uric acid level

For the estimation, serum and double distilled water were taken in an agglutination tube. To it, $2/3(N)$ H_2SO_4 and 10% Na-tungstate were added. The mixture was allowed to precipitate for 10 minutes, filtered and marked as a sample. 400 μ l uric acid was taken as standard and double distilled water was taken as blank in respective microtubes. Na_2CO_3 and uric acid reagent were added in each microtube and were allowed to stand for 15 minutes at room temperature. Then, the optical density (OD) was observed at 630 nm [18].

Serum creatinine level

For the estimation, serum and double distilled water were taken in an agglutination tube. To it, $2/3(N)$ H_2SO_4 and 10% Na-tungstate were added. The mixture was allowed to precipitate for 10 minutes, filtered and later, taken as the sample. 200 μ l creatinine was taken as standard and double distilled water was taken as blank. Picric acid was added to all the tubes and was allowed to stand for 15 minutes. Then, OD was measured at 530 nm [19].

Serum NO

Serum NO levels were determined by following the method of Salkowski *et al.* [20].

Serum pro-inflammatory cytokines

Blood samples from experimental mice were obtained by cardiac puncture at 3, 9, and 15 DPI. The blood (0.2 ml) procured was allowed to clot at 4°C and then centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant, serum was obtained and stored at -80°C for cytokine analysis. Serum from different groups of treated and control mice were normalized to the protein content by Lowry's method before the assay and serum 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.

Synovial tissue enzyme activities

Myeloperoxidase (MPO) assay

It is an indicator of neutrophil infiltration. MPO activity was assayed spectrophotometrically [21].

Lysozyme assay

The assay was performed by taking *Micrococcus lysodeikticus* suspension in a cuvette, and the reaction was started by adding synovial tissue homogenate from each group. The decrease in OD at 450 nm was recorded as a function of time (3 minutes). The change in absorbance for the first 1 minute was used to determine the enzyme activity [22].

Histopathological study

Immediately after the sacrifice of the mice, whole knee joints were removed and fixed for 4 days in 10% formalin. After decalcification in 5% formic acid, all the specimens were processed for paraffin embedding and were sectioned. The tissue sections (7 μ m) were stained with hematoxylin and eosin [23].

Statistical analysis

Results were expressed as mean \pm standard deviation. Statistical analyzes were performed using MS-Office Excel 2007 software packages and one-way ANOVA followed by multiple comparison t-test was performed to find out the significant difference at $p < 0.05$. Correlation coefficients were mentioned as significant both at $p < 0.05$ and $p < 0.001$ [24].

RESULTS

In vivo virulence of three pathogenic *S. aureus* isolates

Table 1 showed that there was a significant increase ($p < 0.05$) in swelling of joints in the pathogenic *S. aureus* (p-1490, p-1488, p-1486) infected mice at 3, 9, and 15 DPI compared with the control.

From Fig. 1, it is observed that the CFU per ml of blood (a) and the CFU per 100 g of infected tissues [spleen (b), synovial tissue (c)] of the strain of *S. aureus* p-1490 infected mice showed significantly higher ($p < 0.05$) number than *S. aureus* p-1488 and *S. aureus* p-1486.

Evaluation of interaction of drugs by checkerboard assay

From the Table 2, it can be observed that the interactions of vancomycin with either AMG or MFA showed indifference in action against the *S. aureus* strain P-1490.

Experimental evaluation of arthritis

The induction of clinical arthritis, calculated from the mean diameters of the wrist and ankle joints of mice, was observed highest in the *S. aureus*-infected group compared to the control group and was highest at 9 DPI which was 55.95% that was seen to be ameliorated maximally in the vancomycin, AMG or MFA co-treated groups, which were 10.48% and 12.11%, respectively (Table 3).

Effect on viable bacteria number in the blood, spleen, and synovial tissue

It was noted after counting the colony from the mannitol salt agar plate that the CFU/ml of blood (Fig. 2a) and CFU/100 mg of spleen (Fig. 2b) in the bacteria challenged mice were highest at 3 DPI, and the CFU/100 mg of synovial tissue (Fig. 2c) was highest at 9 DPI for pathogenic *S. aureus* and then cleared from blood and spleen at day 9 and 15, whereas from synovial tissue at day 15. When AMG or MFA alone was given after 2 hrs of bacterial infection, the bacterial burden was increased in blood and tissues on 3 DPI compared to the non-treated *S. aureus*-infected mice. Treatment with vancomycin alone or co-treatment with either AMG or MFA after infection showed a significant reduction in the CFU count in blood and spleen at 3 DPI while in synovial tissue at 3 and 9 DPI as compared to the infected group ($p < 0.05$).

Effect on blood parameters

Serum uric acid level

Serum uric acid level of *S. aureus* alone infected mice was significantly higher than the control group and was found to be maximum at 9 DPI.

Table 1: Induction of septic arthritis in mice by three strains of *S. aureus* (P-1490, P-1488, P-1486)

Groups	Number of mice	Number of mice died	Paw diameter (mm)			Induction of arthritis (%)		
			Day 3	Day 9	Day 15	Day 3	Day 9	Day 15
Control	6	0	2.81 \pm 0.137	3.09 \pm 0.062	3.06 \pm 0.055	0	0	0
<i>S. aureus</i> (p-1490)	6	1	4.04 \pm 0.175* ψ	4.85 \pm 0.09* ψ	4.06 \pm 0.056* ψ	40.8	58.11	33.44
<i>S. aureus</i> (p-1488)	6	0	3.73 \pm 0.161 ψ	4.31 \pm 0.106 ψ	3.47 \pm 0.04 ψ	30.08	40.47	13.84
<i>S. aureus</i> (p-1486)	6	0	3.56 \pm 0.139 ψ	3.93 \pm 0.118 ψ	3.37 \pm 0.07 ψ	21.37	28.3	10.56

Data of paw diameter are presented as the mean \pm SD, (n=6/group). ψ Control versus all *S. aureus* strains-infected groups; **S. aureus* (1490) versus *S. aureus* (P-1488) and *S. aureus* (P-1486)-infected groups indicate significant difference at $p < 0.05$. *S. aureus*: *Staphylococcus aureus*, SD: Standard deviation

Table 2: Evaluation of synergy against *S. aureus* P-1490 by Checkerboard assay

Drug A	Drug B	Drug C	MICA	MICB	MICC	MICAB	MICBA	MICAC	MICCA	FIC		Inference
										For A+B	For A+C	
Vancomycin	AMG	MFA	2	20	18	2	2	2	2	1.1	1.11	Indifference

Drug A: Vancomycin, Drug B: Aminoguanidine, Drug C: Meclofenamic acid, MIC: Minimum inhibitory concentration, FIC: Fractional inhibitory concentration, *S. aureus*: *Staphylococcus aureus*, MIC: Minimum inhibitory concentrations

Table 3: Effect of treatment of vancomycin alone or in combination with immunomodulators on induction of septic arthritis after *S. aureus* infection in mice

Groups	Number of mice	Number of mice died	Paw diameter (mm)			Induction of arthritis (%)		
			Day 3	Day 9	Day 15	Day 3	Day 9	Day 15
Control	6	0	2.87 \pm 0.163	3.07 \pm 0.054	3.05 \pm 0.041	0	0	0
<i>S. aureus</i>	6	1	4.02 \pm 0.157*	4.79 \pm 0.044*	4.02 \pm 0.046*	40.07	55.95	31.94
S+V	6	0	3.57 \pm 0.067	4.18 \pm 0.043	3.33 \pm 0.084	24.56	36.13	9.332
S+AMG	6	0	3.48 \pm 0.112	3.57 \pm 0.073	3.47 \pm 0.04	21.37	16.31	13.84
S+MFA	6	0	3.53 \pm 0.130	3.61 \pm 0.051	3.40 \pm 0.097	22.82	17.54	11.79
V+S+AMG	6	0	3.32 \pm 0.071**	3.39 \pm 0.037**	3.18 \pm 0.087**	15.85	10.48	4.27
V+S+MFA	6	0	3.25 \pm 0.081**	3.44 \pm 0.051**	3.15 \pm 0.07**	13.39	12.11	3.311

Data of paw diameter are presented as the mean \pm SD, (n=6/group). V: Vancomycin, AMG: Aminoguanidin, MFA: Meclofenamic acid, S: *S. aureus*. *Control versus *S. aureus*; ***S. aureus* versus combination treatment groups indicate significant difference at $p < 0.05$. *S. aureus*: *Staphylococcus aureus*, SD: Standard deviation

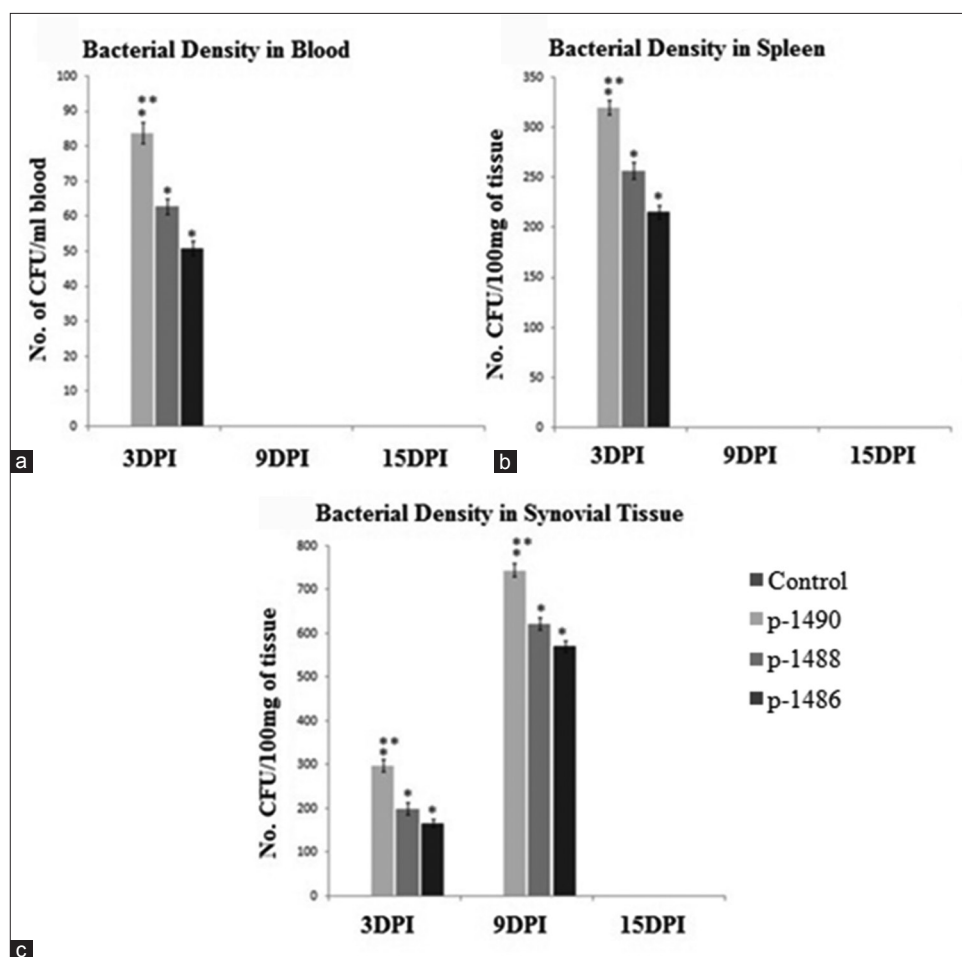


Fig. 1: Bacterial density in blood (a), spleen (b), and synovial tissue (c) of *Staphylococcus aureus* (P-1490, P-1488, and P-1486) infection in mice at 3, 9, and 15 days post-infection. Data expressed as mean \pm standard deviation ($n=6$ /group); *Control versus all *S. aureus* strains-infected groups; ***S. aureus* (1490) versus *S. aureus* (P-1488) and *S. aureus* (P-1486)-infected groups indicate significant difference at $p<0.05$

Treatment of mice with vancomycin, AMG or MFA alone after infection showed significant reduction in serum uric acid level at 3, 9, and 15 DPI than *S. aureus* alone infected mice ($p<0.05$), but maximum reduction was found in the group of mice co-administered with vancomycin, AMG or MFA after being infected with *S. aureus* ($p<0.05$) (Fig. 3a and c). Serum uric acid level showed statistically significant correlation with induction of arthritis in *S. aureus*-infected mice on 3 DPI ($r=0.951$, $p<0.001$), 9 DPI ($r=0.974$, $p<0.001$), and 9 DPI ($r=0.948$, $p<0.05$), but no significant correlation in vancomycin and AMG or MFA co-administered group ($p>0.05$).

Serum creatinine level

S. aureus-infected mice showed significantly higher serum creatinine level than the control group at 3, 9 as well as 15 DPI. However, the treatment of mice with vancomycin or AMG or MFA alone after infection significantly reduced the serum creatinine level and was further reduced by the combination treatment of vancomycin with either AMG or MFA ($p<0.05$) (Fig. 3b and d). Serum creatinine levels also presented significant correlation with induction of arthritis in *S. aureus*-infected mice on 3 DPI ($r=0.951$, $p<0.001$), 9 DPI ($r=0.974$, $p<0.001$), and 9 DPI ($r=0.948$, $p<0.05$), but no significant correlation was observed in vancomycin and AMG or MFA co-administered mice ($p>0.05$).

Serum NO level

Serum NO level significantly increased in only *S. aureus*-infected group as compared to the control and significantly decreased from the

infected group, in the group of mice that obtained AMG treatment alone as well as the group that obtained both vancomycin and AMG treatment after being infected with *S. aureus* ($p<0.05$). However, no significant reduction was observed in MFA-treated groups of mice (Fig. 4).

Pro-inflammatory cytokine levels

Serum levels of TNF- α , IFN- γ , and IL-6 were elevated significantly after *S. aureus* infection ($p<0.05$) and significant diminution were observed in all the treatment groups maximum being in the mice treated with vancomycin with either AMG or MFA ($p<0.05$). However, vancomycin, AMG, or MFA alone and also vancomycin in combination with either AMG or MFA could significantly up-regulate serum IL-10 at 3, 9, and 15 DPI ($p<0.05$) (Fig. 5).

Synovial tissue enzyme activities

MPO activity

The MPO activity is an indicator of neutrophil infiltration, and was observed to be significantly elevated in the *S. aureus*-infected group ($p<0.05$) on 3, 9, and 15 DPI. It showed statistically significant correlation with induction of arthritis on 3 DPI ($r=0.967$, $p<0.001$), 9 DPI ($r=0.988$, $p<0.001$), and 9 DPI ($r=0.859$, $p<0.05$). When vancomycin or AMG or MFA was administered alone following *S. aureus* infection, it produced a significant reduction of tissue MPO activity, but maximum diminution was found in the group of mice that co-treated with vancomycin, AMG, or MFA after *S. aureus* infection ($p<0.05$) (Fig. 6a and c). It was also reflected in the induction of arthritis, which

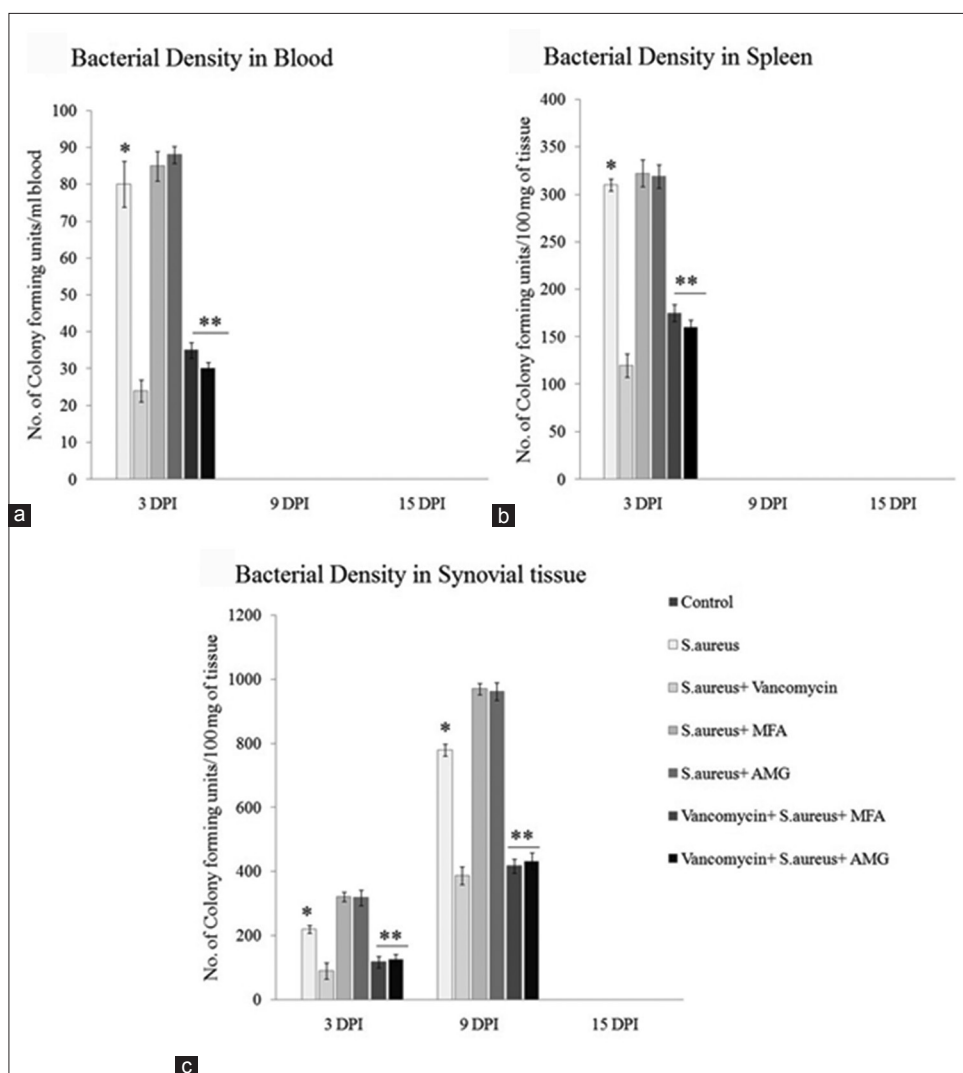


Fig. 2: Effect of vancomycin alone or in combination with immunomodulators on bacterial density in blood (a), spleen (b), and synovial tissue (c) of *Staphylococcus aureus* infection-induced septic arthritis. Data expressed as mean \pm standard deviation (n=6/group); *Control versus *S. aureus*; ***S. aureus* versus combination treatment groups indicate significant difference at $p < 0.05$

showed no significant correlation with MPO activity in vancomycin and AMG or MFA co-administered group ($p > 0.05$).

Lysozyme activity

Synovial tissue lysozyme activity in the *S. aureus*-infected mice was significantly higher than the control group at 3, 9, and 15 DPI ($p < 0.05$) and was highest at 9 DPI. The mice treated with vancomycin or AMG or MFA alone after *S. aureus* infection showed significant decrease in lysozyme activity but the utmost reduction in the same was found in the group that was treated with vancomycin along with AMG or MFA after being infected with *S. aureus* ($p < 0.05$) (Fig. 6b and d). Lysozyme activity also showed significant correlation with induction of arthritis in *S. aureus*-infected groups on 3 DPI ($r = 0.951, p < 0.001$), 9 DPI ($r = 0.974, p < 0.001$), and 9 DPI ($r = 0.948, p < 0.05$), but no significant correlation in vancomycin and AMG or MFA co-administered group ($p > 0.05$).

Histopathological examination

Histological examination of the synovial joint showed pronounced tissue degradation, erosion of bones and the articular cartilage as well as narrowing of joint spaces in the group only infected with *S. aureus*, whereas, in the group treated with vancomycin along with either AMG or MFA showed much less signs of synovitis (Fig. 7).

DISCUSSION

Acute *Staphylococcal* septic arthritis is a pathological inflammatory emergency because bacterial replication in the joint with the inflammatory processes can lead to rapid local joint destruction which may be accompanied by systemic infection. Moreover, with the emergence of MRSA, alternative therapeutic measures to combat *S. aureus*-induced arthritis has become a prerequisite. Numerous antibiotics have activity against MRSA, but published documentations with these drugs are limited to case reports and observational studies. Hence, the present investigation was undertaken with antibiotic vancomycin, in an attempt to mitigate *S. aureus* burden, along with the co-administration of immunomodulators, AMG or MFA, which are efficacious in inhibiting endogenous NO [25] and PG [26], respectively, to treat *Staphylococcal* arthritis in mice model. The checkerboard assay shows that the efficacy of mitigation of *S. aureus* by vancomycin is not diminished or accelerated by either of the immunomodulators, AMG or MFA (Table 2). The MIC of vancomycin for *S. aureus* P-1490 was determined to be 2 $\mu\text{g/L}$ whose nearly 10 times dose (23 mg/kg body weight of mice) was administered as calculated from the equation mentioned earlier and vancomycin being a concentration dependent antibiotic, it often works best at almost 10 times its MIC [27].

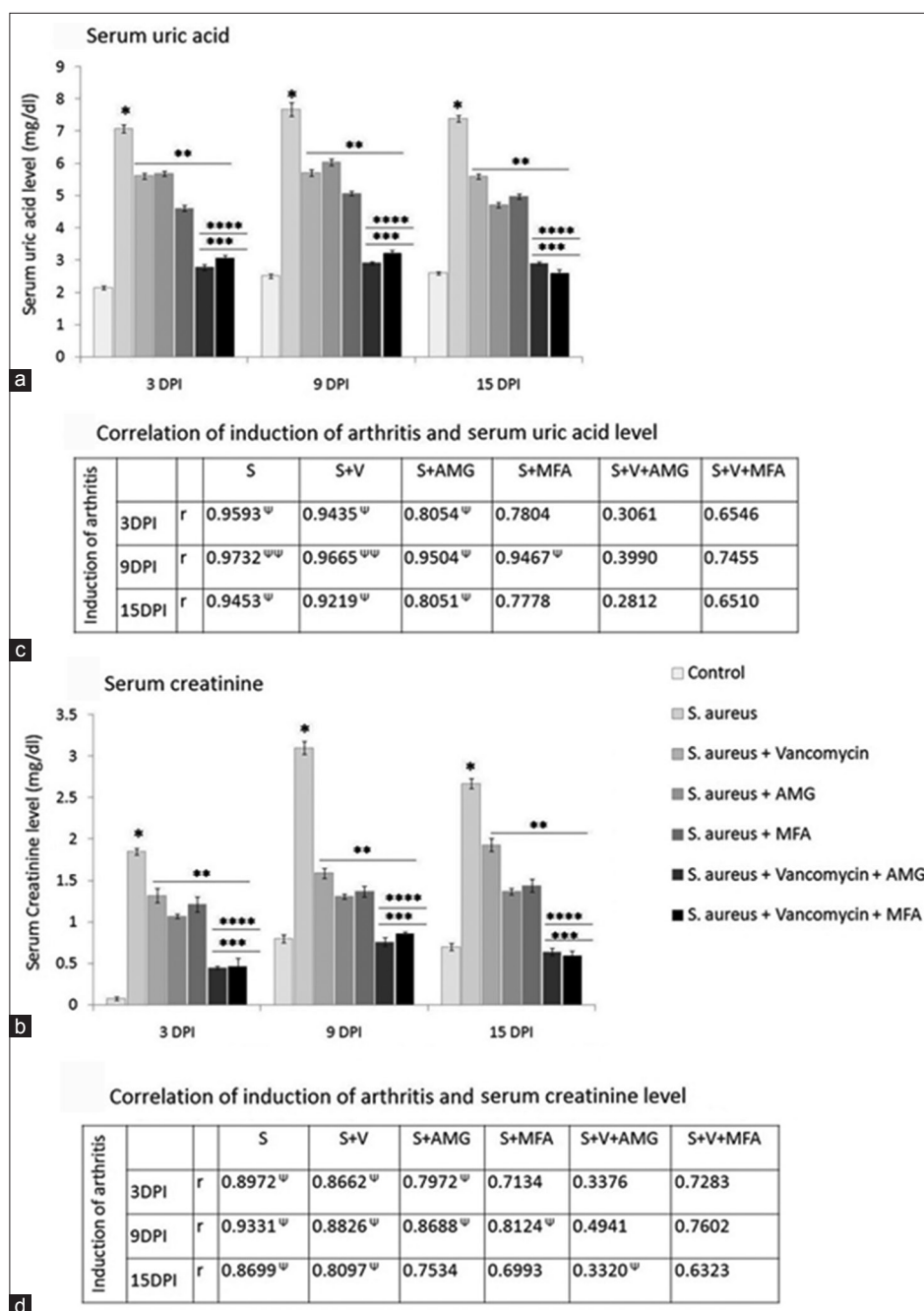


Fig. 3: Serum uric acid (a) and creatinine (b) levels in *Staphylococcus aureus*-infected mice after treated with vancomycin alone or in combination with immunomodulators. Correlation of induction of arthritis with serum uric acid (c) and creatinine (d) level in *S. aureus*-infected mice after treated with vancomycin alone or in combination with immunomodulators. Data expressed as mean ± standard deviation (n=6/group); *Control versus *S. aureus*; ***S. aureus* versus treated groups; ***(*S. aureus* + vancomycin) versus (*S. aureus* + vancomycin + aminoguanidin [AMG]) and (*S. aureus* + vancomycin + meclofenamic acid [MFA]); ****(*S. aureus* + AMG) or (*S. aureus* + MFA) versus (*S. aureus* + vancomycin + AMG) or (*S. aureus* + vancomycin + MFA) indicate significant difference at p<0.05; correlation co-efficient (r) shows significant differences at^ψp<0.05 and^{ψψ}p<0.001

The *S. aureus* that had been injected through the tail vein of mice must have reached the synovial joints via the hematogenous route, grew themselves, and triggered immune responses. From the periodic assessment of bacterial clearance from the tissues and blood of the mice, that were infected separately with the three strains of *S. aureus* (P-1490, P-1488, or P-1486) (Fig. 1); as well as the paw diameter measurement and the percentage of induction of arthritis (Table 1) in mice by these three *S. aureus* strains showed that among the three acquired hospital strains of *S. aureus* (P-1490, P-1488, P-1486), the strain *S. aureus* P-1490 is the most virulent and so it has been used to pursue the study.

Thus, in the study, to actualize the effect of vancomycin in combination with either AMG or MFA in the modulation of septic arthritis in mice, mice were infected with *S. aureus* P-1490 followed by the combination treatments. Here, the CFU count from the blood, spleen, and synovial tissue and the observations from the arthritic scorings might have been successful in depicting the distribution of the bacterial burden with respect to induction of arthritis (Table 3). Clearing of the bacterial colonies from blood and spleen from 9 DPI may point out the migration of *S. aureus* into the synovial tissues by then, which may also be inferred from the rise in bacterial densities in the synovial tissue at 9 DPI and

maximum percentage of induction of arthritis in synovial tissue at 9 DPI in the *S. aureus*-infected group. Thus, bacterial density was consequently more in the group which received only *S. aureus* infection compared to the control group. The *S. aureus*-infected group which received only AMG or MFA showed even higher density of bacteria as compared to the group only infected by *S. aureus*, perhaps giving a picture of the antibacterial role of endogenous NO and PG, whose synthesis when blocked by administration of AMG (iNOS inhibitor) and MFA (COX-2 inhibitor), respectively, leads to weakening of host immune defence to mitigate bacterial colonization but could reduce inflammatory outcome to some extent which could be inferred from reduction in the percentage of arthritis induction in these groups (Table 3). Whereas, the groups treated by vancomycin in combination with either AMG or MFA after being infected with *S. aureus* showed reduction in bacterial density compared to the only *S. aureus*-infected group as well as maximum abatement of arthritic induction percentage, probably enabling to perceive that the combination treatment might have been potent in mitigation of bacterial burden and also in nullifying inflammatory agony to appreciable extent (Fig. 2).

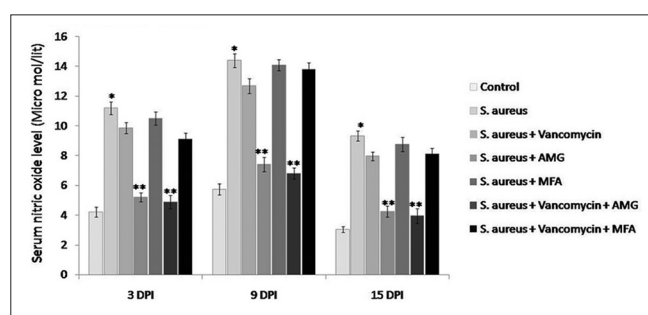


Fig. 4: Vancomycin alone or in combination with immunomodulators on serum nitric oxide levels of *Staphylococcus aureus*-induced septic arthritis. Data expressed as mean \pm standard deviation (n=6/group); *Control versus *S. aureus*; *S. aureus* versus *S. aureus* + aminoguanidin (AMG) and *S. aureus* + vancomycin + AMG, indicate significant difference at $p < 0.05$**

The above mechanism, in turn, may have stimulated mast cells, complement factors, macrophages along with other immune counterparts [28] resulting in release of pro-inflammatory mediators such as cytokines, TNF- α , IL-6, and IFN- γ whose serum levels were detected to be highest in *S. aureus*-infected group, decreasing in vancomycin-treated and infected group due to reduced bacterial colonies and further reduction in AMG or MFA-treated and infected group, being least in the *S. aureus*-infected group which received combination therapy of vancomycin with AMG or MFA (Fig. 5). These presumably activated gene transcription factors such as nuclear factor-kappa B, signal transducer, and activator of transcription-1 [29]. Cytokines could also be triggered via interaction of pathogenic components with pattern recognition receptors or via the immunoglobulin E immune complexes which conceivably could activate COX-2 and iNOS promoter. The activated iNOS with nicotinamide adenine dinucleotide phosphate and L-arginine might generate citrulline and NO. The serum level of NO was found to be elevated in *S. aureus*-infected group while down regulated in the groups that obtained AMG treatment (Fig. 4). NO, besides being a pro-inflammatory substance by itself, also possesses the ability to induce elevation of levels of other pro-inflammatory mediators such as TNF- α and IFN- γ [30]. The interaction of host immune responses and bacterial components might propel reactive oxygen species (ROS) which in combination with NO might have brought forth peroxynitrite (ONOO⁻) and other reactive nitrogen species (RNS) into action [31]. NO and RNS most likely could bring about synovial tissue and articular cartilage disruptions, narrowing of joint spaces, neutrophil migration into joint spaces, activation of other immune weapons accentuating inflammation, which this study intended to highlight.

Out of miscellaneous molecular pathways aiding anti-inflammatory actions during septic arthritis, we opted to focus on IL-10 as emblematic of cytokines in this class. The primary site of inflammation, serum, was examined to obtain IL-10 level which continued to increase even at 15 DPI in the *S. aureus*-infected groups that were treated by vancomycin in combination with AMG or MFA (Fig. 5). This increment might be accepted as a positive prognostic sign of recovery from the inflammatory severity of septic arthritis, due to the mentioned combination therapy. IL-10 may have restricted the generation of ROS and RNS, which were the outcome of IFN- γ activation of monocyte or macrophages. Thereby,

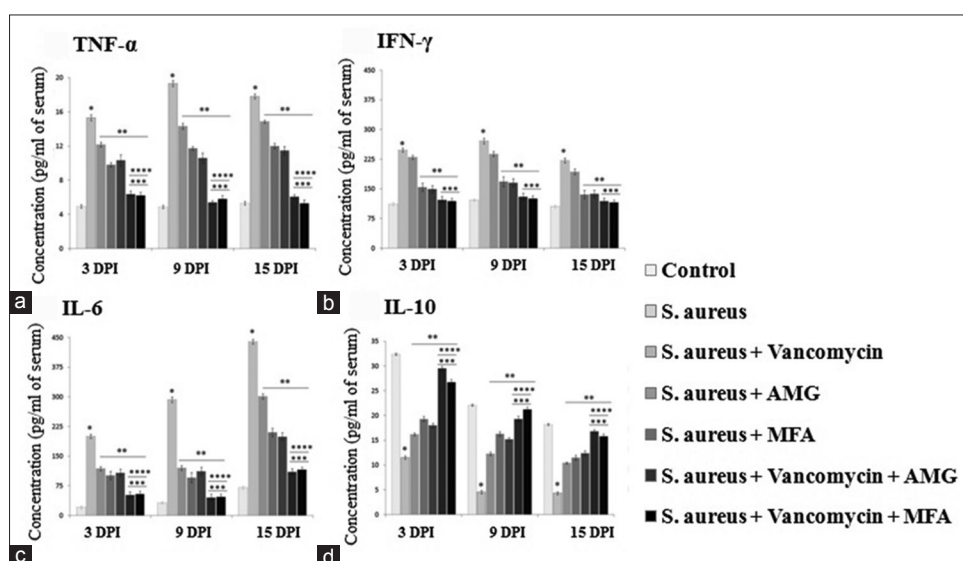


Fig. 5: Effect of vancomycin alone or in combination with immunomodulators on serum tumor necrosis factor alpha (a), interferon gamma (b), interleukin 6 (IL-6)(c), and IL-10 (d) of *Staphylococcus aureus* infection-induced septic arthritis. Data expressed as mean \pm standard deviation (n=6/group); *Control versus *S. aureus*; *S. aureus* versus treated groups; ****S. aureus* + vancomycin versus *S. aureus* + vancomycin + aminoguanidin (AMG) and *S. aureus* + vancomycin + meclofenamic acid (MFA); *****S. aureus* + AMG or *S. aureus* + MFA versus *S. aureus* + vancomycin + AMG or *S. aureus* + vancomycin + MFA indicate significant difference at $p < 0.05$**

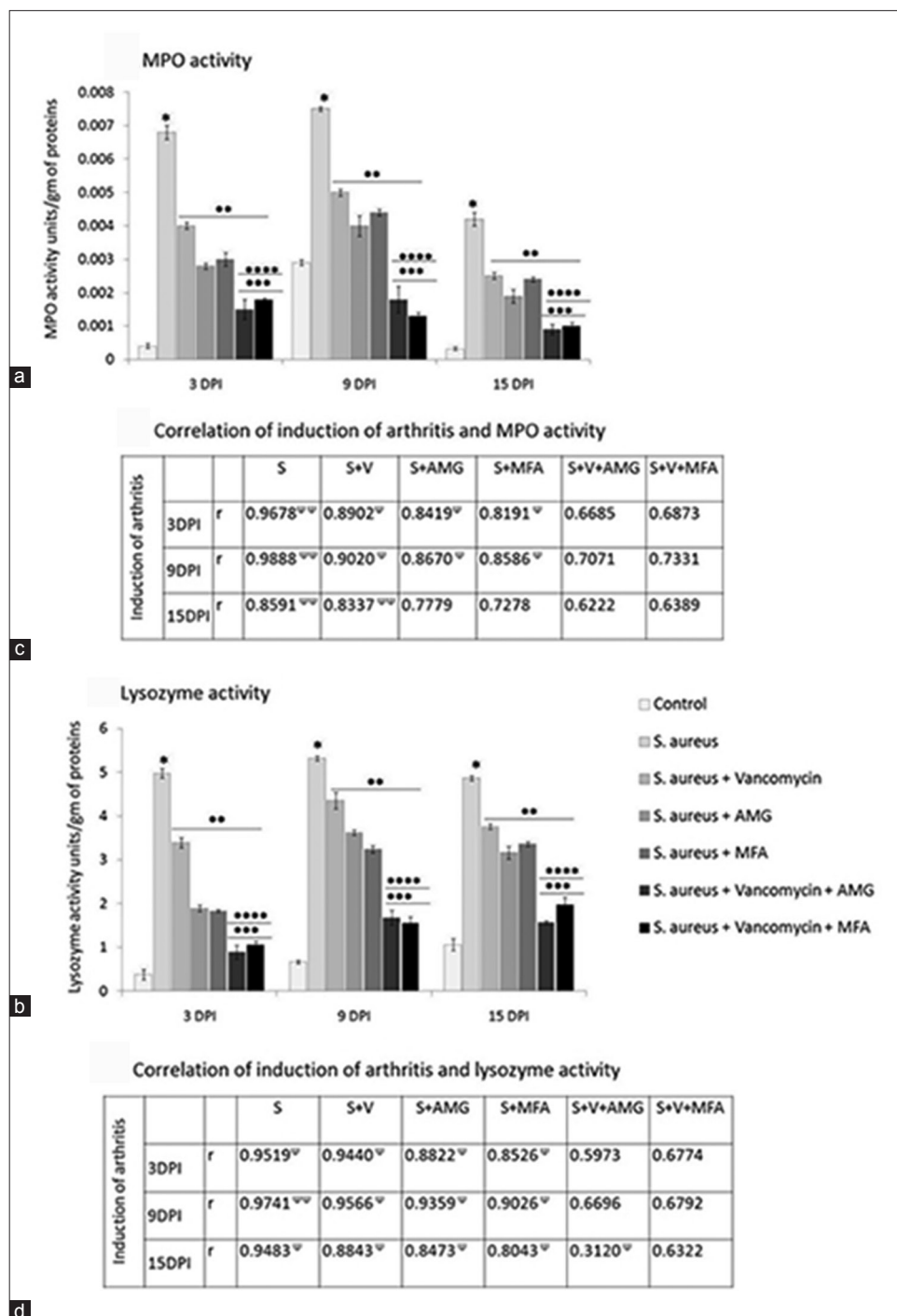


Fig. 6: Myeloperoxidase (MPO)(a) and lysozyme (b) activities in *Staphylococcus aureus*-infected mice after treated with vancomycin alone or in combination with immunomodulators. Correlation of induction of arthritis with MPO (c) and lysozyme (d) activities in *S. aureus*-infected mice after treated with vancomycin alone or in combination with immunomodulators. Data expressed as mean \pm standard deviation (n=6/group); *Control versus *S. aureus*; ***S. aureus* versus treated groups; ****S. aureus* + vancomycin versus *S. aureus* + vancomycin + aminoguanidin (AMG) and *S. aureus* + vancomycin + meclufenamic acid (MFA); *****S. aureus* + AMG or *S. aureus* + MFA versus *S. aureus* + vancomycin + AMG or *S. aureus* + vancomycin + MFA indicate significant difference at p<0.05; correlation co-efficient (r) shows significant differences at [∇]p<0.05 and ^{∇∇}p<0.001

the combination treatment perhaps attempts to prevent exaggeration of the inflammatory situation.

On the other hand, the percentage of induction of arthritis in different groups of mice has been found to change according to the biochemical sequel that followed the onset of inflammation. Serum uric acid and creatinine are the key clinical manifestations of inflammation. The

mechanism by which uric acid, the “pathogenic culprit” promotes inflammation, that is, by an endogenous adjuvant or by stimulating IL-1 β or via activation of the nucleotide oligomerization domain-like receptor protein, is still under investigation [32]. Serum creatinine level is an indicator of tissue protein breakdown as inflammation proceeds. In this study, both the serum uric acid and creatinine distinctly get elevated in the group that is being infected with *S. aureus* as compared to the control

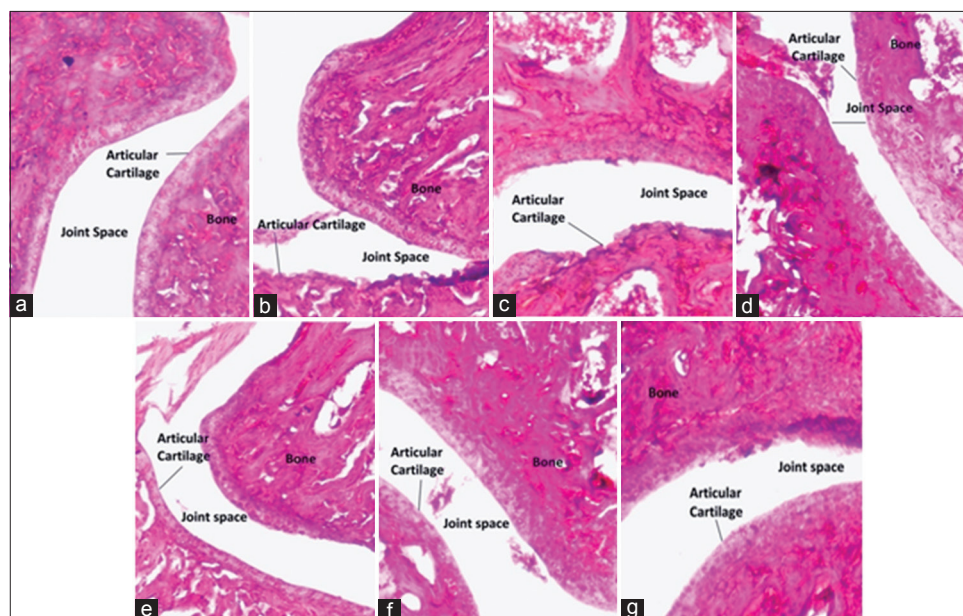


Fig. 7: Photomicrographs of paraffin-embedded H and E-stained rat knee-joint sections ($\times 200$) of control mice (a) treated with *Staphylococcus aureus* (b) treated with *S. aureus* and vancomycin (c) treated with *S. aureus* and aminoguanidin (AMG) (d) treated with *S. aureus* and meclufenamic acid (MFA) (e) treated with *S. aureus*, vancomycin and AMG (f) and treated with *S. aureus*, vancomycin and MFA (g), respectively

group assuming that the *S. aureus*-infected group may be inflamed to the most critical level (Fig. 3). When AMG or MFA, administered alone in the *S. aureus*-infected group, serum uric acid, and creatinine showed a lower level of occurrence than the only infected group and; but, when administered with vancomycin, the serum uric acid, and creatinine were reduced almost to the control level. Vancomycin alone did not reduce the serum uric acid or creatinine level to that extent perhaps due to lack of proper penetration of the antibiotic into the inflammation struck synovial tissues. Thus, impeding the endogenous NO and PG synthesis, the inflammatory asperity may be constructively modulated and, on combining this with the antibacterial activities of vancomycin, whose action might have been facilitated by the co-administration of the immunomodulators, further diminution in clinical parameters such as serum uric acid and creatinine may be obtained.

The activated COX-2 conceivably has produced PGs from arachidonic acid and other pro-inflammatory mediators may augment vascular permeability. NO and PGs permit infiltration of neutrophils, lymphocytes, and propagation of inflammatory conditions, and thus, the infiltrated macrophages and neutrophils show MPO and lysozyme activities to fight against bacterial survival worsening the septic scenario [33]. Significantly heightened actions of lysozyme and MPO in *S. aureus*-infected synovial tissue and its curtailed activities after administration of AMG or MFA demonstrate the anti-inflammatory role of immunomodulators (Fig. 6). The reduced lysozyme and MPO activity in synovial tissue may suggest that AMG or MFA and vancomycin probably inhibit the inflammatory responses by depreciating neutrophil infiltration into the synovial tissue and effectively killing the homed *S. aureus*, respectively.

The septic scenario may result in undesirable changes in the synovial joint morphology which is depicted by the histological study. The articular damage and narrowing of the joint spaces are almost nullified in the *S. aureus*-infected group that obtained treatment of vancomycin, which is assumed to be more efficient than other antibiotics due to its low protein binding, in combination with AMG and MFA (Fig. 7).

CONCLUSION

It may be assumed from this study that AMG or MFA, via their anti-inflammatory puissance, may abate the severity of inflammatory up

shots in septic arthritis. However, prevention of these immunological responses leads to increasing load of *S. aureus* density in the synovial tissue, whose mitigation might be achieved by the assistance of vancomycin treatment, in combination with the above-mentioned agents. Evaluation of the level of endogenous PG level, as well as insights into the molecular level to assess the mRNA expressions of iNOS and COX-2, might further enrich the findings of this study. Basic research with a proper understanding of the biological mechanisms involved in a specific infection might help in higher therapeutic researches to combat infectious diseases. Therefore, the outcome of this study may suggest that the remedy to septic arthritis caused by *S. aureus* may be achieved to a certain extent by the combination treatment of vancomycin with either AMG or MFA and might encourage more advanced research to successfully accomplish strategy to combat septic arthritis, one of the most agonizing maladies with soaring occurrence.

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