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# Role of plasminogen activator inhibitor – 1 (PAI-1) in regulating the pathogenesis of *S. aureus* arthritis via plasminogen pathway

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ABSTRACT

uPA/tPA-mediated activation of plasminogen/plasmin pathway during *S. aureus* arthritis facilitates the invasion of phagocytes in the affected joint, induces production of cytokines and triggers inflammatory pathways. PAI-1, an effective inhibitor of both uPA and tPA, attenuates plasmin activity. Hence, the objective of our study was to evaluate the effect of exogenously administered PAI-1 on uPA/tPA-mediated activation of plasminogen/plasmin and its impact on the progression of arthritis. The mice were infected with live *S. aureus* and treated with PAI-1. Mice were sacrificed at 3, 9 and 15 days post infection and thereafter assessment of parameters related to arthritic destruction was done. PAI-1 administration resulted into decrement in uPA and tPA activities with a concomitant reduction in plasmin and MMP-2. A significant decrement in the joint and paw swelling with lower levels of inflammatory cytokines, RANKL and OPN activities were detected in case of early PAI-1 treatment. This study suggests administration of PAI-1 during *S.aureus* arthritis reduces the severity of arthritis by ameliorating uPA and plasmin-induced inflammatory responses and subsequent arthritic destruction.

## 1. Introduction

*Staphylococcal* arthritis is usually induced by bacteremia causing synovial inflammation and bone-cartilage destruction leading to permanent reduction in joint function [1]. The plasminogen/plasmin system has been reported to play an important role during the development of different forms of arthritis [2–4]. Two plasminogen activators, tissue-type PA (tPA) or urokinase-type PA (uPA) cleave the inactive plasminogen to its active form, plasmin [5]. According to different published reports, uPA and its receptor, uPAR contribute to arthritis pathogenesis by mediating local inflammatory reactions in the affected joint [6]. Hence, u-PA can be recognized as an essential mediator of joint inflammation [7,8]. Interestingly, t-PA−/− mice display more severe disease pathogenesis when compared to its relevant wild-type mice indicating a protective role of tPA in arthritis [9].

The activation of plasminogen to plasmin by uPA/tPA can be involved in several processes like inflammatory cells activation, removal of necrotic tissues, and cytokine releases. Although these processes are essential to fight against the invading pathogens, further destruction can be ensured if these uncontrolled responses continue for a longer duration [10]. The absence of arthritis in the plasminogen/plasmin-deficient mice suggests a further contribution of the plasminogen/plasmin system in the development of arthritis that encourages the exploration of strategies to inhibit plasmin activity during arthritis [11]. Plasminogen activator inhibitor-1 (PAI-1) is the principal inhibitor of both t-PA and u-PA [12]. Although PAI-1 inhibits both uPA and tPA, the inhibitory effect of PAI-1 is more prevalent on uPA than tPA [13].

Plasmin and uPA promote MMP-2 activation, a crucial factor responsible for ECM (extracellular matrix) degradation and progression of septic arthritis [14]. Infection-induced hyperactivation of MMP-2 causes degradation of ECM at the level of the joint [15,16]. PAI-1 can possibly inhibit MMP-2 activation directly [17] and indirectly by impeding uPA and plasmin formation.

Inflammation is generally accompanied by bone erosion. Osteoclastogenesis or formation of osteoclast is essential for bone

*Abbreviations:* CFU, colony forming unit; DPI, days post infection; ECM, extracellular matrix; IP, intraperitoneal; MCP-1-Monocyte, chemoattractant protein-1; MMP-2, matrix metalloproteinase-2; MPO, myeloperoxidase; NO, nitric oxide; $O_2^{\cdot}$-, superoxide anion; OPG, osteoprotegerin; OPN, osteopontin; PAI-1, plasminogen activator inhibitor-1; PBS, phosphate buffered saline; RANKL, receptor activator of nuclear factor kappa-B ligand; ROS, reactive oxygen species; *S. aureus*, *Staphylococcus aureus*; tPA, tissue-type plasminogen activator; TNF-α, tumor necrosis factor- alpha; TRAP, tartarate acid phosphatase; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor

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destruction and erosion [18]. Osteopontin (OPN), an extracellular matrix protein, appears to influence osteoclastic activity responsible for bone resorption [19,20]. RANKL, a member of TNF superfamily, binds to the RANK receptor expressed on osteoclastic precursor and stimulates the differentiation of osteoclasts. Osteoprotegerin (OPG) acts as a decoy receptor of RANK that inhibits the RANKL/RANK interaction [21]. OPG knockout mice are reported to be osteoporotic due to deregulated RANKL/RANK interaction and increased osteoclast formation within synovial tissue [22]. According to established reports, plasmin has some dramatic influences on the expressions of OPG and RANKL suggesting plasmin-mediated bone resorption [23]. As plasmin-mediated cleavage of OPN is required for its activation, therefore OPN could also be a novel substrate for plasmin [24]. Hence, it will be interesting to figure out the effect of exogenous PAI-1 administration on bone destruction and matrix degradation via the plasminogen/plasmin pathway.

Several reports suggest the pathogenic contribution of PAI-1 in different diseases such as diabetes, renal fibrosis, myocardial infarction and atherosclerosis [25,26]. Interestingly exogenous administration of PAI-1 has been found to be effective in preventing hypoxic - ischemic brain injury [27] and hepatic fibrosis [28]. No reports regarding the impact of PAI-1 during *S.aureus* induced arthritis has been available so far. We, therefore, hypothesized that exogenous administration of PAI-1 during *S. aureus* arthritis might regulate uPA/tPA mediated conversion of plasminogen to plasmin which in turn could modulate the level of inflammatory cytokines, extracellular matrix degradation and the process of bone destruction.

## 2. Materials and methods

### 2.1. Experimental animals

Adult Swiss albino male mice (6–8 weeks of age with body weight 20 ± 4 g) were used for this experimental study. Mice were kept under the controlled environment (temperature 23 ± 2 °C and 50 ± 5% humidity with a 12-h light-dark cycle) and fed a normal rodent diet. Mice were approved by the Institutional animal ethical committee (IAEC), (Approval Number: IAEC/IV/Proposal/BB-02/2015 dated 23.11.2015).

The animals (n = 10/group) were grouped into i) Control (CON) – 3 DPI, ii) Control (CON) – 9 DPI, iii) Control (CON) – 15 DPI, iv) Control + treatment with Plasminogen Activator Inhibitor – 1 without *S. aureus* infection (CON + PAI-1) - 3 DPI, v) Control + treatment with Plasminogen Activator Inhibitor – 1 without *S. aureus* infection (CON + PAI-1) - 9 DPI, vi) Control + treatment with Plasminogen Activator Inhibitor – 1 without *S. aureus* infection (CON + PAI-1) - 15 DPI, v) *S. aureus* infected alone (SA) - 3 DPI, vi) *S. aureus* infected alone (SA) - 9 DPI, vii) *S. aureus* infected alone (SA) - 15 DPI, viii) *S. aureus* infected + treatment with Plasminogen Activator Inhibitor – 1 (SA + PAI-1) - 3 DPI ix) *S. aureus* infected + treatment with Plasminogen Activator Inhibitor – 1 (SA + PAI-1) - 9 DPI. x) *S.aureus* infected + treatment with Plasminogen Activator Inhibitor – 1 (SA + PAI-1) - 15 DPI. The whole experimental set up was repeated twice.

### 2.2. Preparation of bacteria

The bacterial strain used in this study was *Staphylococcus aureus* (*S. aureus* - strain # AG-789, a toxic shock syndrome toxin-1 positive strain). This strain had been extensively used in our previous mouse models of septic arthritis [29–31]. Bacteria were cultured, harvested, washed in sterile phosphate buffered saline (PBS), and adjusted to the desired inoculums spectrophotometrically before infection (Optical Density$_{620}$ = 0.2 = $5 \times 10^7$ cells/ml for *S. aureus*) [32].

### 2.3. Induction of arthritis

*S. aureus* inoculum suspended in 100 µl of sterile phosphate buffered saline (PBS) was intravenously injected into the mouse tail vein ($5 \times 10^6$cells/mouse of average body weight of 20gm). Control mice received 100 µl of sterile PBS. This model has been successfully used in our previous works [29–31].

### 2.4. Treatment of S. aureus infected mice with plasminogen activator inhibitor – 1 (PAI-1)

PAI-1, (Abcam, Catalog number - ab142349) dissolved in saline at a dose of 2.5 nmol/kg of body weight, was injected (100 µl) intraperitoneally after 24 h of infection and continued up to 15 days at a regular interval (day 1, day 4, day 7, day 10 and day 13). The dose of PAI-1 used in this study was found to be effective and safe in the mice model [33]. We further did a dose-response trial (pilot study) to determine the optimal but nonlethal dose of PAI-1 required for the inhibition of plasminogen activators (Determination of PAI-1 activity during this experiment was not done.).

### 2.5. Assessment of septic arthritis

Arthritis induction was assessed by measuring the synovial knee joint and paw swelling on a daily basis with a dial-type vernier caliper. For the knee joint swelling, the daily mean values for each of the groups were determined by the number of synovial knee joints measured in each group. These average values represented the severity of knee joint swelling [34]. Percentage induction or reduction in arthritis per group was calculated as follows:

[(Mean diameter of swelling of synovial knee joints on day 15 – Mean diameter of swelling of synovial knee joints on day 15 after treatment)/Mean diameter of swelling of synovial knee joints on day 15] × 100.

For the paw swelling, the clinical severity of arthritis was graded on a scale of 0–3 for each paw, according to changes in erythema and swelling. 0 - no change; 1 - mild swelling and/or erythema; 2 - moderate swelling and erythema; 3- marked swelling, erythema, and/or ankylosis. Thus, a mouse could have a maximum score of 12. The arthritis index (mean ± SD) was constructed by dividing the total score (cumulative value in all paws) by the number of animals used in each experimental group [35].

### 2.6. Sacrifice of animals

Mice were anesthetized with ketamine hydrochloride (Sigma, Life Sciences) at a dose of 1 mg/kg body weight via the tail vein at 3, 9 and 15 dpi. Collection of blood (0.5 ml) was done by cardiac puncture followed by isolation of spleens and synovial tissues. The whole experimental design has been elaborately explained in Fig. 1.

### 2.7. Determination of the number of viable S. aureus cells in blood, spleen and synovial tissue

Spleen tissues and joint tissues were weighed and homogenized in sterile RPMI-1640 medium (3 ml/100 mg spleen tissue and 1 ml/100 mg joint tissue) immediately after each sacrifice. All the homogenized tissue samples and blood samples were plated in triplicate on mannitol agar and the results were expressed as the number of Colony Forming Units (CFU) per gm of tissue or per ml of blood respectively. To avoid false positive results due to contamination, an isolate was considered positive when 15 or more *S. aureus* colonies were present [36].

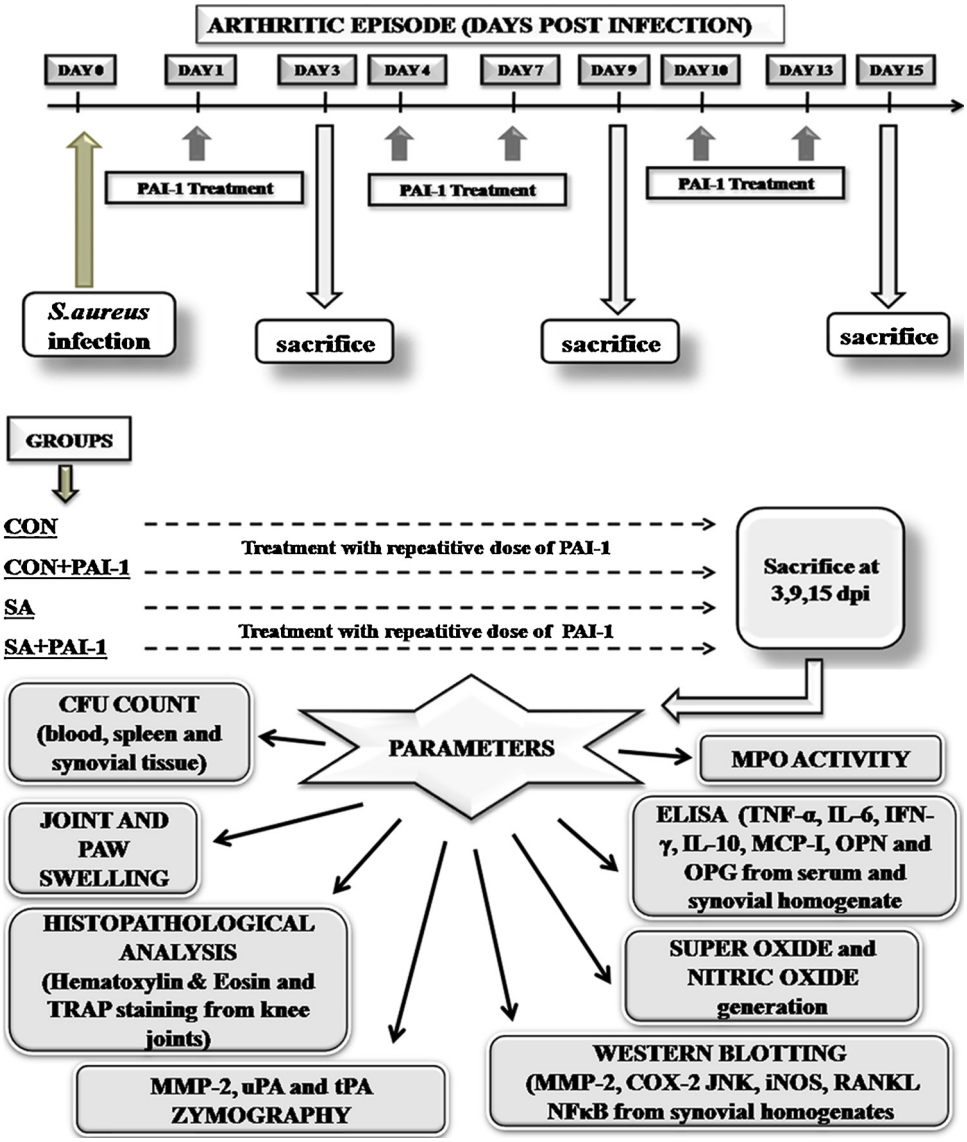


**Fig. 1.** Design of work: Treatment with PAI-1 during different days post *S. aureus* infection.

### *2.8. Hematoxylin eosin staining of synovial knee joints for histological analysis*

Samples of whole knee joints were collected for histological analysis. Briefly, the knee joints were fixed in 4% paraformaldehyde, decalcified in decalcification buffer (10% EDTA, 7.5% polyvinylpyrrolidone, 0.1*M* Tris, adjusted to pH 6.95) for 14 days at room temperature and 8-μm paraffin-embedded sections were prepared. Joint tissue sections were stained with hematoxylin and eosin. For each mouse, both knee joints were considered.

Cell infiltration in the synovial joint space was graded from 0 to 3; 0 = no inflammatory cells in the joint. 1 = few inflammatory cells in the joint, 2 = joint cavity partially filled with inflammatory cells, 3 = joint cavity totally filled with inflammatory cells. Tissue necrosis was graded from 0 to 3; 0 = no necrotic tissue, 1 = few necrotic tissues, 2 = joint cavity filled with necrotic tissue, 3 = joint cavity totally filled with necrotic tissues. Synovitis was graded from 0 to 3; 0 = un inflamed appearance of synovium, 1 = mild thickening of the synovium, 2 = moderate thickening of the synovium, 3 = severe thickening of the synovium. Destruction of bone and cartilage was graded from 0 to 3; 0 = normal appearance, 1 = minor sign of destruction, 2 = moderate loss of the bone and cartilage, 3 = severe loss of bone and cartilage [37].

### *2.9. Quantification of TRAP (tartarate acid phosphatase) positive cells in the synovial joint and cartilage-bone interface by TRAP staining*

At first tissue sections were deparaffinised. After that TRAP staining was performed using a commercial acid phosphatase leucocyte kit (Sigma, St Louis, MO). Five areas (magnification × 40) were randomly observed in the synovial joints and the cartilage-bone interfaces. The number of TRAP positive cells in each area was counted [38].

### *2.10. Plasminogen activators zymography*

To assess uPA and tPA activities, 50 μg of synovial tissue lysate were incubated with non-reducing buffer for 20 min at 37 °C and then electrophoresed in a polyacrylamide gel containing 2 mg/ml α-casein (Sigma-Aldrich) and 5 μg/mL plasminogen. Gels were washed 2 times for 30 min each in 50 mM Tris-HCl (pH 7.6) and 2.5% Triton X-100. Then the gels were incubated for 16 h at 37 °C in 50 mM Tris-HCl (pH 7.6) followed by staining in a solution of 30% methanol, 10% acetic acid and 0.5% Coomassie brilliant blue G250 for 1 h. Next de-staining was done. The activities of uPA and tPA were detected as digested clear

bands on a blue background [39]. Gels were scanned with Adobe Photoshop version 7.0 and visualized as black and white images. The intensity of each band was determined by densitometry (Biorad gel documentation system, Image J software) and the units were expressed as relative fold change in uPA or tPA activity compared to the control.

### 2.11. Determination of plasmin concentration

The plasmin activity was determined by hydrolysis of the specific plasmin substrate (D-Val-Leu-Lys 4-nitroanilide dihydrochloride – Sigma Catalog no – V0882). The rate of change in the absorbance was measured spectrophotometrically at 405 nm [40].

### 2.12. Extraction and analysis of MMP-2 by gelatin zymography

The protein concentration in the samples was determined by the Lowry method [41]. Twenty micrograms of soluble proteins from synovial knee joint tissue samples were diluted with nonreducing sample buffer. Then the samples were separated in a 7.5% polyacrylamide gel containing 1 mg/ml of gelatin. The gel was subjected to electrophoresis at 20 mA for 90 min, Next it was incubated in 2.5% Triton X-100 Tris buffered saline for 60 min and 20 h at 37 °C in buffer containing 100 mM CaCl2 followed by staining with 0.2% Coomassie. The gel was scanned with Adobe Photoshop version 7.0 and visualized as a black and white image. The intensity of each band was determined by densitometry (Biorad gel documentation system, Image J software) and the unit was expressed as relative fold change in MMP-2 activity compared to the control [42].

### 2.13. Western blot analysis for expressions of MMP-2, NF-κB, JNK, RANKL, cyclo oxygenase– 2 (COX-2) and inducible nitric oxide synthase (iNOS) from synovial tissues

The synovial joints from different groups of mice were homogenized in RIPA buffer and centrifuged at 10,000 rpm for 10 min. The protein concentrations were measured by the Lowry method before the gel loading. Proteins were denatured at 100 °C for 5 min in the SDS-PAGE loading buffer. The aliquots containing an equal amount of total protein from each sample were separated by SDS-PAGE (10% gel). After the separation, proteins in the slab gel were transferred onto nitrocellulose transfer membranes. After blocking for 2 h the membranes were washed three times in Tris-buffered saline and Tween-20 (TBST) and probed overnight at 4 °C with the appropriate primary antibodies separately (MMP-2, NF-κB, JNK, RANKL, COX-2 and iNOS.) (MMP-2: Biorbyt UK catalog no. Orb101824; NF-κB: Biorbyt UK catalog no. Orb11118, JNK: Biorbyt UK catalog no. Orb14628; RANKL: Biorbyt UK catalog no. Orb6560; COX-2: Orb106537; iNOS Biorbyt UK catalog no. Orb13614). The membranes were washed three times in TBST. Next it was incubated for 2 h with horse radish peroxidase (HRP) conjugated secondary antibodies and developed with chemiluminescent substrate [43]. Densitometric data were expressed as arbitrary units normalized on the expression of the protein β –tubulin.

### 2.14. Sample preparation for cytokines TNF-α, IL-6, IFN-γ, IL-10, osteopontin, osteoprotegerin and MCP-1 measurement

The blood samples were kept at 4 °C for 45 min followed by centrifugation at 3000 rpm for 5 min at 4 °C. The serum was thus separated and after that, the protein contents were determined and normalized by the Lowry method. The levels of TNF-α, IL-6, IFN-γ and IL-10 were assayed using ELISA kits strictly on next day after each sacrifice (TNF-α, IL-6, IFN-γ, IL-10, osteopontin, osteoprotegerin and MCP-1 from RayBiotech. Inc). For each study, the cytokines levels were determined in duplicate, in a single run to avoid inter-assay variability, and intra-assay variability. The minimum detectable level of TNF-α, IL-6, IFN-γ, IL-10, osteopontin, osteoprotegerin and MCP-1 was 60 pg/ml, 5 pg/ml, 2 pg/ml, 45 pg/ml, 4 mg/ml, 1 mg/ml and 3 mg/ml respectively. The reproducibility of cytokine kits is as follow; for intra-assay: CV < 10%, for interassay: CV < 12%.

### 2.15. Myeloperoxidase (MPO) enzyme activity

The MPO enzyme activity is an index of neutrophil infiltration in the synovial tissue. MPO activity was determined from the synovial tissues according to the protocol used in the previously published reports [29–31]. The rate of changes in the absorbance was measured spectrophotometrically at 405 nm. MPO enzyme activity was considered as the concentration of the enzyme degrading 1 μM of peroxide/min at 37 °C [44].

### 2.16. Assessment of superoxide anion ($O_2^{\cdot -}$) and nitric oxide (NO) production in synovial tissue

In response to the inflammatory stimuli induced by *S. aureus*, activated phagocytes generate higher levels of reactive oxygen species (ROS) including superoxide anion ($O_2^{\cdot -}$) and nitric Oxide (NO) in the affected synovial joint. The assessments of superoxide and nitric oxide were done as described previously [45,46]. The amount of superoxide anion production was calculated by the following formula: *Micromoles of superoxide anion = (mean absorbance at 550 nm × 15.87)**

The amounts of NO produced were determined by extrapolation from a standard curve prepared in parallel using sodium nitrite.

### 2.17. Statistical analysis

All the results were expressed in means ± SD (n = 6/group). The Assessment of significant differences between the groups was performed using a one-way analysis of variance (ANOVA). A Scheffe's F-test posthoc test for multiple comparisons of the different groups was done when significant p-values were obtained. All the analyses were done using the Origin Pro 8 software (Origin Lab Corporation, Northampton, MA). All the data were normally distributed and the distribution is confirmed by the Shapiro -Wilk test.

## 3. Results

### 3.1. Changes in the knee joint and paw swelling of the S. aureus infected mice after treatment with PAI-1: experimental evaluation of arthritis

Knee joint swelling was evaluated to determine the severity of arthritis in the mice belonging to different groups. The swelling was significantly increased in the *S. aureus* infected group (SA) at 3, 9 and 15 dpi when compared to the control ones (CON). A significant reduction in the joint swelling was observed at 3 and 9 dpi after administration of exogenous PAI-1 in the infected group (SA + PAI-1) ($P < 0.05$). However, at 15 dpi no significant change in the joint swelling was detected ($P < 0.05$) in the SA + PAI-1 group compared to the *S.aureus* infected alone (SA) (Table 1).

Similar to the joint swelling, arthritis index calculated from the paw swelling of the mice also indicated significant decrement in the paw swelling from day 2 to day 13 after PAI-1 treatment in the infected mice (SA + PAI-1) when compared to only *S. aureus* infected (SA) mice ($P < 0.05$). Importantly, Maximum arthritis index of 2.85 ± 0.298 and 1.88 ± 0.124 were determined on day 15 in the SA and SA + PAI-1 groups respectively (Fig. 2A).

### 3.2. Body weight and mortality in mice belonging to different groups

Body weight and mortality of the mice were strictly checked at regular time intervals throughout the experiment. No significant alteration in body weight and mortality was obtained among different groups of mice (hence data not shown).

**Table 1**
Experimental evaluation of arthritis (joint swelling): Effect of administration PAI-1 in *S. aureus* infection induced arthritis. The severity of arthritis was evaluated regularly by experimental assessment of swelling of the joint tissues. Values are expressed as mean ± SD from each group at different dpi.

| | Average knee joint diameter in mm (Mean ± SD) GROUPS | | | | Percentage Induction of arthritis (%) in SA group compare to CON group | Percentage Reduction of arthritis (%) in SA + PAI-1 group compare to SA group |
|---|---|---|---|---|---|---|
| DPI | CON | CON + PAI-1 | SA | SA + PAI-1 | | |
| 3 | 2.10 ± 0.22 | 2.22 ± 0.31 | 4.20 ± 0.40* | 2.86 ± 0.312*,# | 50 | 31.90 |
| 9 | 2.01 ± 0.24 | 2.12 ± 0.12 | 4.86 ± 0.52* | 3.02 ± 0.325*,# | 58.64 | 37.86 |
| 15 | 2.03 ± 0.29 | 2.05 + 0.18 | 4.98 ± 0.62* | 4.53 ± 0.335 | 59.23 | 9.03 |

* indicates significant difference in comparison to CON ($P < 0.05$).
\# indicates significant difference in comparison to SA ($P < 0.05$).

### 3.3. Bacterial burden in the infected mice treated with PAI-1

The growth of bacteria was found in the joints, spleens and blood of all the infected groups at 3 dpi. The bacterial load was present in the joints (Fig. 2B) and spleens (Fig. 2C) but not in the blood at 9 dpi (Fig. 2D). No bacterium was detected either in blood, spleen or synovial tissue samples at 15 dpi in any group. SA + PAI-1 group showed significant deduction in the bacterial burden at 3 and 9 dpi in case of spleen and synovial samples only ($P < 0.05$) when compared to the SA group.

### 3.4. Histopathological findings and osteoclastogenesis by TRAP staining at 3 and 9 days after PAI-1 administration in infected mice

Histopathological analysis (Fig. 3A–K) revealed a drastic reduction in cellular infiltration and synovitis with less degradation of bone and cartilage in SA + PAI-1 in comparison with the mice infected with SA alone at 3 and 9 dpi but not at 15 dpi. However, tissue necrosis was not altered significantly after PAI-1 treatment in any dpi. Tissue necrosis was observed to be maximum at 15 dpi (Fig. 3I). According to the TRAP staining images, TRAP positive cells were also decreased significantly ($P < 0.05$) after PAI-1 treatment at 3 and 9 dpi but not at 15 dpi

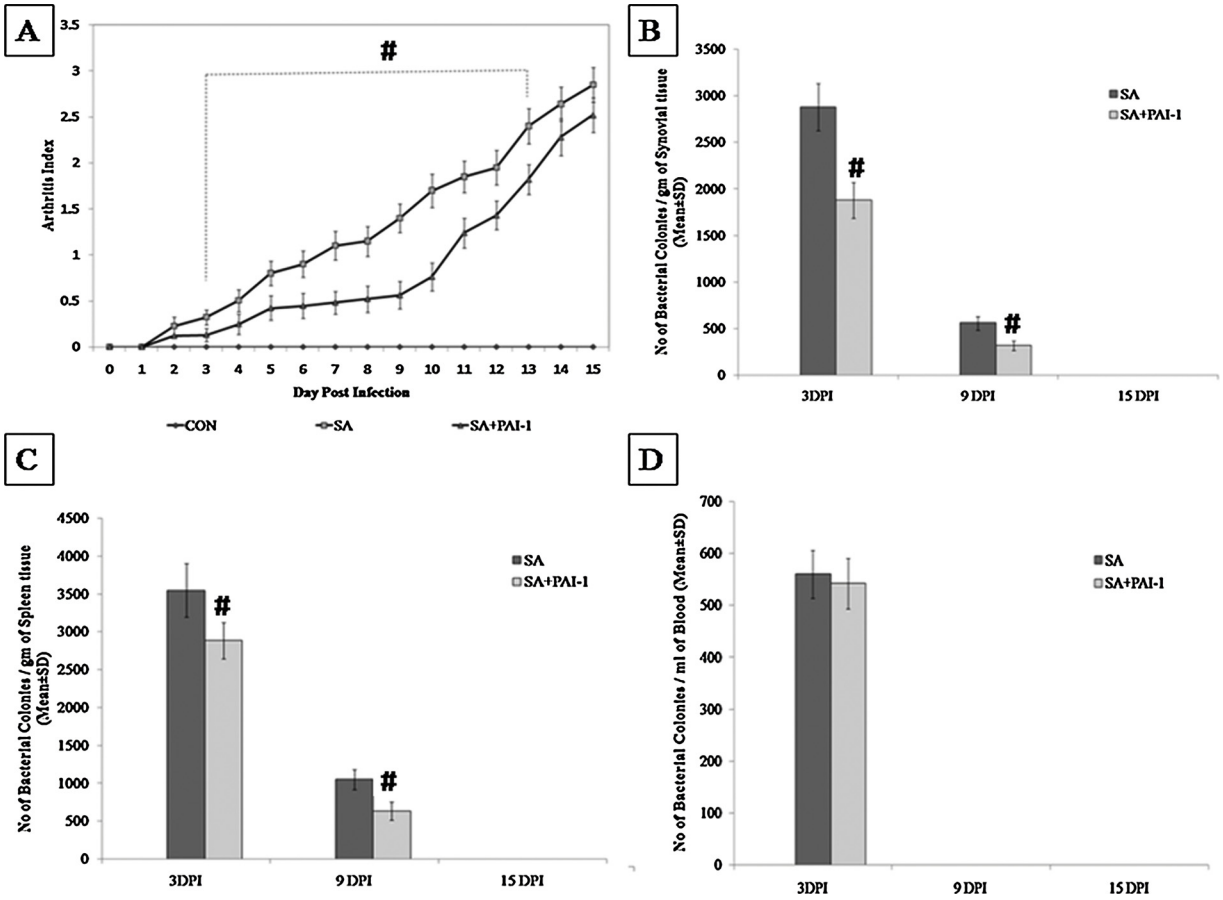


**Fig. 2.** (A) Experimental evaluation of arthritis (paw swelling) and effect of PAI-1 administration on the bacterial burden in synovial tissue (B), spleen (C) and blood (D) in arthritic mice: (A) Arthritis index was evaluated in CONTROL, SA and SA + PAI-1 groups. (B–D) Bacterial load was determined from synovial tissue (B), spleen (C), and blood (D) of infected mice during 3, 9 and 15 dpi. At 9 dpi bacterial count was negligible in blood (positive count ≥ 15). At 15 dpi no bacteria was detected in any sample. Values are expressed as mean ± SD from each group at different dpi. * indicates significant difference in comparison to CON ($P < 0.05$). # indicates significant difference in comparison to SA ($P < 0.05$).

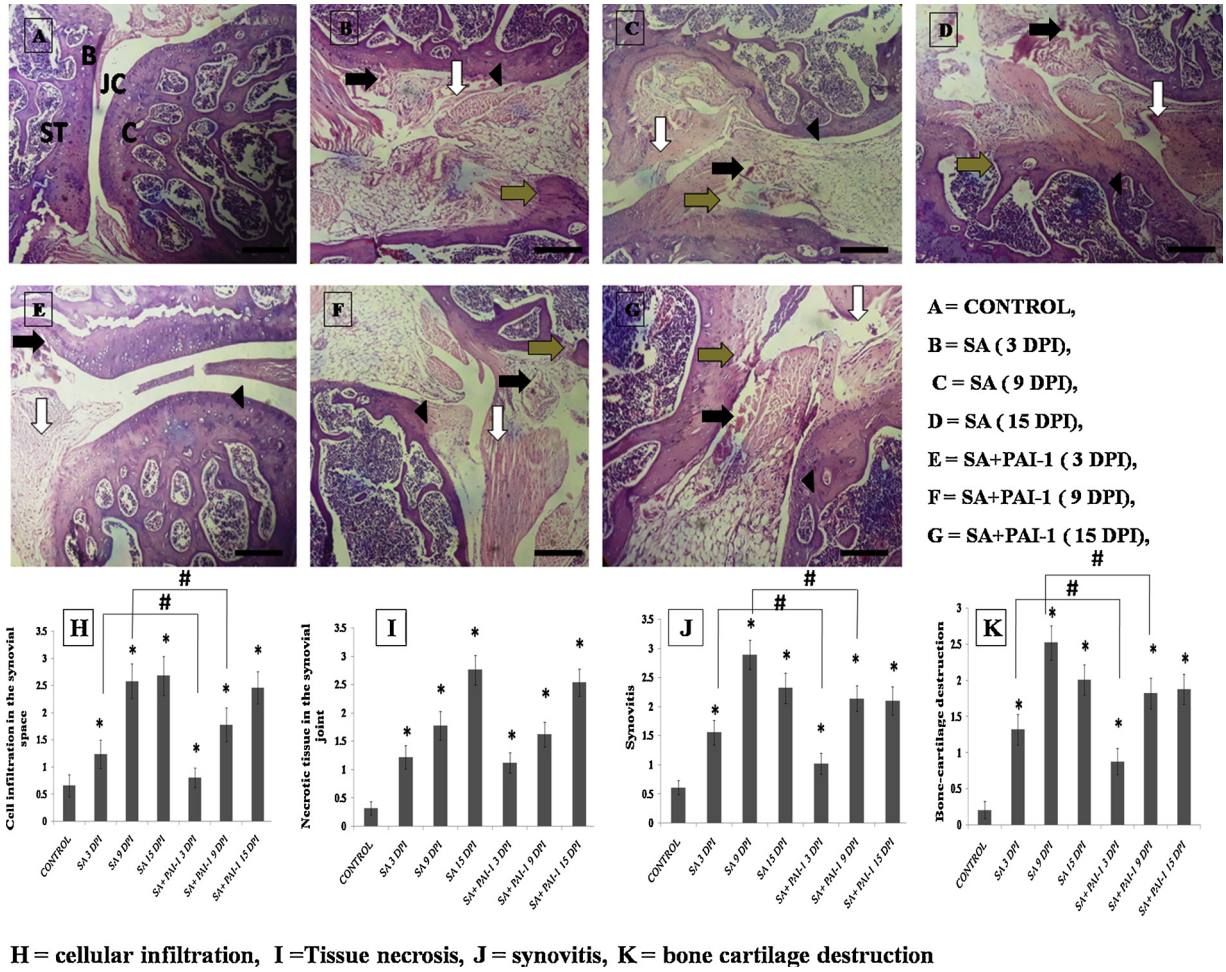


**Fig. 3.** Hematoxylin-Eosin staining of arthritic synovial knee joint at bone cartilage interface: Hematoxylin-Eosin staining images of arthritic joints of mice belonging to different groups during different days post infection. Cellular infiltration (black arrows) necrosis (black arrow heads) synovitis- pannus (white arrows) and bone cartilage destruction (grey arrows) (Bar = 200μm.). A = Control, B = *S. aureus* (3 DPI), C = *S. aureus* (9DPI), D = *S. aureus* (15 DPI), E = *S.aureus* + PAI-1 (3 DPI), F = *S.aureus* + PAI-1 (9 DPI), G = *S.aureus* + PAI-1 (15 DPI). H = Bar diagram representing cellular infiltration, I = Bar diagram representing necrotic tissues, J = Bar diagram representing synovitis, K = Bar diagram representing bone cartilage destruction. B = bone, C = cartilage, ST = synovial tissue and JC = joint cavity. * indicates significant difference in comparison to CON ($P < 0.05$). # indicates significant difference in comparison to SA ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

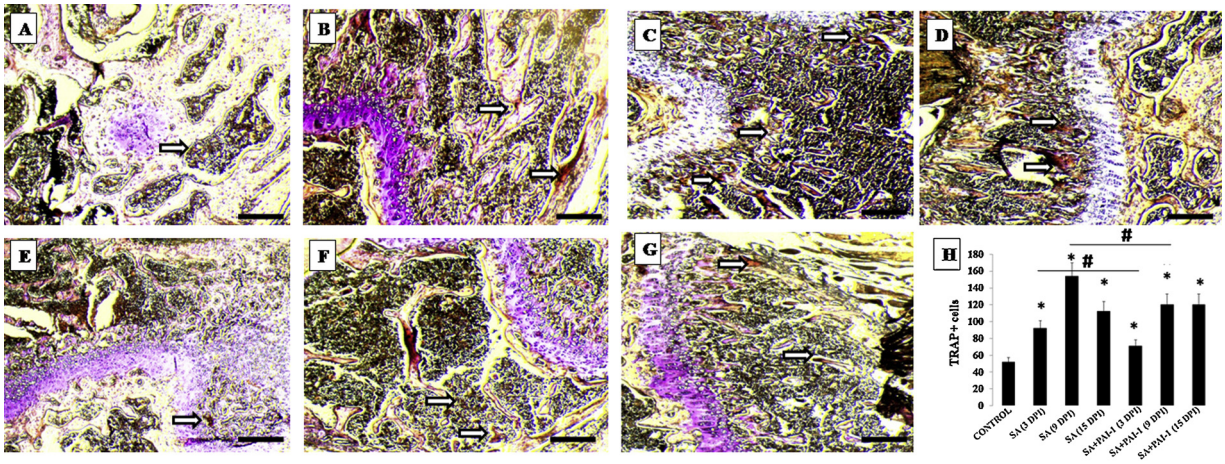


**Fig. 4.** TRAP staining of arthritic synovial knee joint at bone cartilage interface: TRAP staining images of arthritic joints of mice belonging to different groups. Black arrows indicating TRAP positive cells (Bar = 100μm.). A = Control, B = *S. aureus* (3 DPI), C = *S. aureus* (9DPI), D = *S. aureus* (15 DPI), E = *S.aureus* + PAI-1 (3 DPI), F = *S.aureus* + PAI-1 (9 DPI), G = *S. aureus* + PAI-1 (15 DPI). H = Bar diagram representing TRAP positive cells counts from TRAP staining of tissue sections belonging to different groups. * indicates significant difference in comparison to CON ($P < 0.05$). # indicates significant difference in comparison to SA ($P < 0.05$).

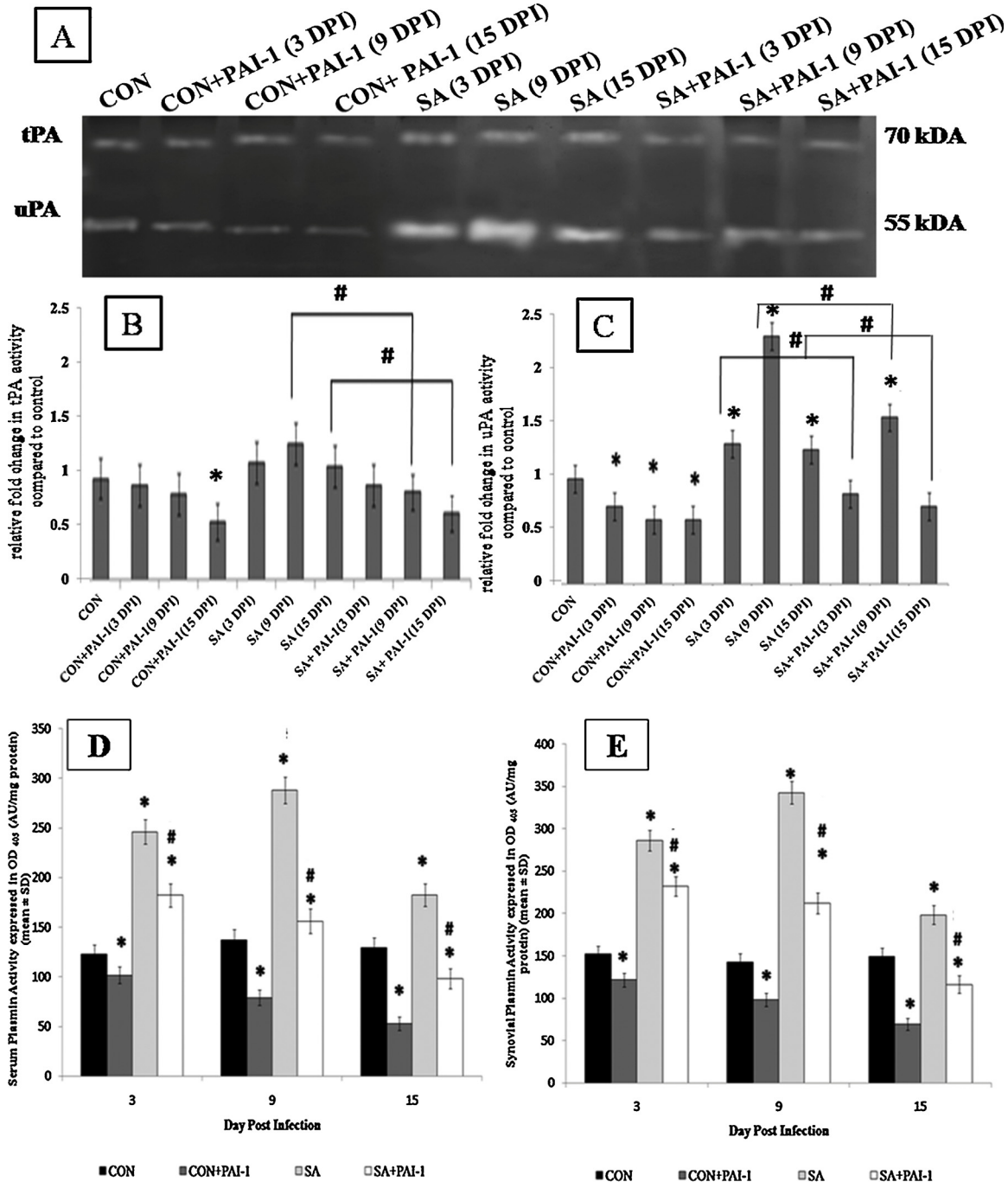


**Fig. 5.** (A–C): Casein zymography analysis of uPA and tPA activation and (D–E) changes in plasmin activity at 3, 9, 15 days post *S. aureus* infection in synovial joints. (A) Expressions of uPA and tpA were observed at 3, 9, 15 days post infection in CON, CON + PAI-1, SA and SA + PAI-1 groups of mice. (B) Fold changes in uPA activity during 3, 9, 15 dpi in different groups of mice. (C) Fold changes in tPA activity during 3, 9, 15 dpi in different groups of mice. The units are expressed as relative fold change in uPA or tPA activity compared to control. Bar diagrams indicating changes in plasmin activity in serum (D) and synovial tissue homogenates (E) at different days post infection in CON, CON + PAI-1, SA and SA + PAI-1 groups of mice. Values are expressed as mean ± SD from each group at different dpi. * indicates significant difference in comparison to CON ($P < 0.05$). # indicates significant difference in comparison to SA ($P < 0.05$).

(Fig. 4A–H) ($P > 0.05$).

### 3.5. uPA and tPA activities in synovial tissues of PAI-1 administered groups

The casein zymography was done to determine the impact of PAI-1 on uPA and tPA activities during the progression of septic arthritis (Fig. 5A). The activity of uPA increased markedly under the arthritic condition with maximum activity at 9 dpi in *S. aureus* infected mice ($P < 0.05$). The tPA activity did not alter significantly due to *S. aureus* infection when compared with the control ones ($P > 0.05$). Inhibition of uPA at 3, 9 and 15 dpi (Fig. 5C) and inhibition of tPA at 9 and 15 dpi (Fig. 5B) were clearly depicted ($P < 0.05$) in the groups treated with PAI-1 in *S.aureus* infected animals (SA + PAI-1).

### 3.6. Plasmin activity in the serum and the synovial knee joints of the arthritic mice after treatment with PAI-1

The activity of plasmin was monitored in the serum (Fig. 5D) and

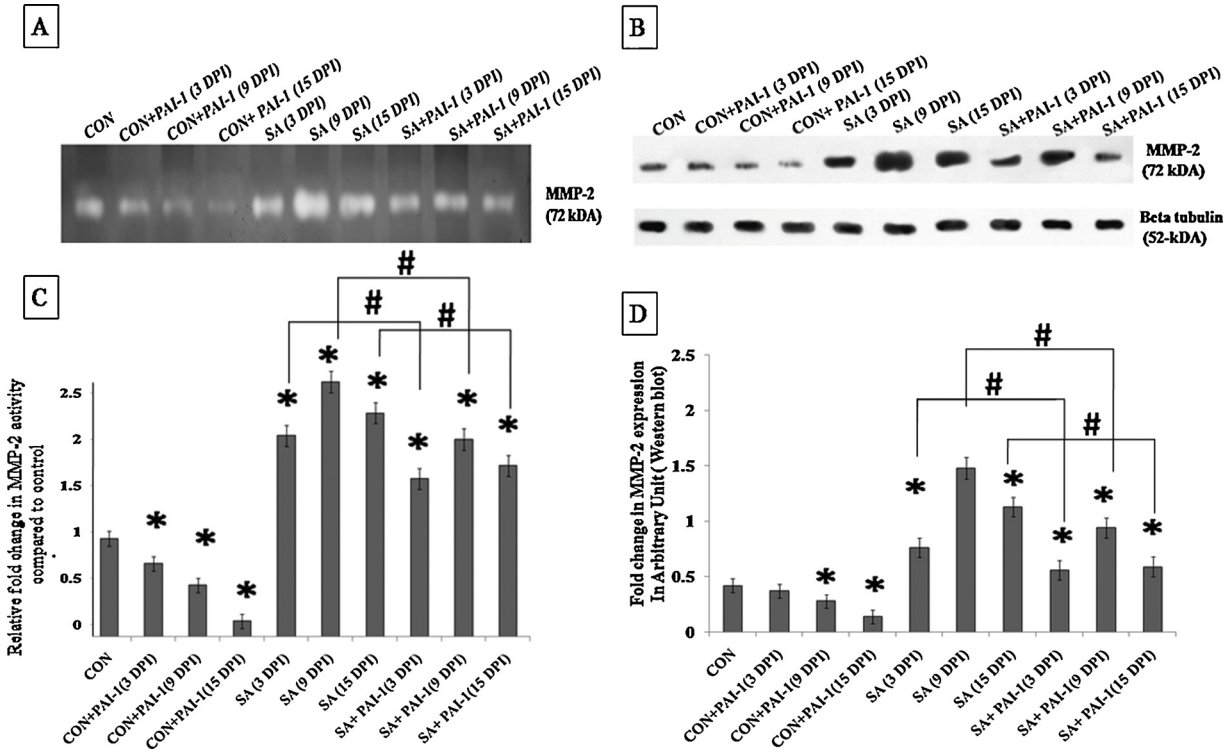


**Fig. 6.** (A) Gelatin zymography (A, C) and western blot analysis of MMP-2 (B, D) at 3, 9, 15 days post *S. aureus* infection from synovial joints: Change in MMP-2 expression (A,C) was observed in synovial tissue homogenates at different days post infection in CON, CON + PAI-1, SA and SA + PAI-1 groups of mice Expression of MMP-2 by western blot (B, D) at different days post infection was detected in CON, CON + PAI-1, SA and SA + PAI-1 groups of mice. The units are expressed as relative fold change in MMP-2 activity compared to control. Values are expressed as mean ± from each group at different dpi. * indicates significant difference in comparison to CON ($P < 0.05$). # indicates significant difference in comparison to SA ($P < 0.05$).

the synovial tissue (Fig. 5E) to determine the effect of PAI-1 in the conversion of plasminogen to its active form plasmin. High serum and synovial plasmin activities were detected in *S. aureus* infected mice signifying infection induced plasmin activation. The plasmin activity was dramatically reduced both in the serum and synovial samples ($P < 0.05$) as a result of PAI-1 administration with the maximum reduction at 15 dpi (even lower than control level at 15 dpi).

### 3.7. PAI-1 administration induced changes in MMP-2 expression in the arthritic mice

MMP-2 activity (Fig. 6A) and expression (Fig. 6B) in the joint samples remained remarkably higher at 3, 9 and 15 dpi in *S. aureus* infected mice ($P < 0.05$) according to the data obtained from gelatin zymography and western blotting respectively. Maximum MMP-2 activity and expression were detected at 9 dpi in the infected group. Administration of PAI-1 after infection (SA + PAI-1) caused a marked reduction in MMP-2 activity and expression (Fig. 6C and D) at 3, 9 and 15 dpi ($P < 0.05$) than *S. aureus* infected alone (SA).

### 3.8. Effect of PAI-1 treatment on pro inflammatory cytokines and chemokine MCP-1 in the serum and the synovial tissue during the different phases of arthritis

According to the ELISA reports, pro inflammatory cytokines such as TNF-α (Figs. 7A & 8 A), IL-6 (Figs. 7B & 8 B) and IFN-γ (Figs. 7C & 8 C) remained significantly elevated in *S.aureus* infected group of mice both at local (serum; Fig. 7) and systemic (synovial tissue; Fig. 8) levels ($P < 0.05$). TNF-α reached its peak at 9 dpi both in serum and synovial samples of SA. IL-6 reached its highest level at day 9 in the serum and at day 15 in the synovial tissue of the *S.aureus* group ($P < 0.05$). Both local and systemic IFN-γ concentrations remained significantly increased at 9 and 15 dpi in the infected mice. The serum and synovial IL-10 (Figs. 7D & 8 D) was noticed to be higher in *S.aureus* infected mice at day 3 when compared to day 9 and day 15. At 15 dpi production of these cytokines was more prominent in the *S.aureus* infected synovial tissue than the serum. The highest level of chemokine MCP-1 (Figs. 7E & 8 E) was found at 9 dpi both in the serum and synovial samples of *S.aureus* infected mice when compared to the control group ($P < 0.05$). Treatment with exogenous PAI-1 in the *S.aureus* infected mice (SA + PAI-1) showed significant lowering in the serum and the synovial levels of TNF-α, IL-6, IFN-γ and MCP-1 at 3 ($P < 0.05$) and 9 dpi ($P < 0.01$) but not at 15 dpi ($P > 0.05$). Noteworthy, PAI-1 administration did not alter the concentration of serum or synovial IL-10 levels in *S.aureus* infected mice ($P > 0.05$).

### 3.9. Expressions of NF-κB, JNK, iNOS, COX-2 and RANKL in the arthritic knee joints of infected mice after administration of PAI-1 at 3 and 9 dpi

*S. aureus* infected mice (SA) were found to have significantly higher levels of NF-κB (Fig. 9, B), JNK (Fig. 9, C), iNOS (Fig. 9, D), COX-2 (Fig. 9, E), and RANKL (Fig. 9, F) expressions in synovial joints compared to the control ones. Treatment with exogenous PAI-1 after infection (SA + PAI-1) expressed decreased NF-κB, JNK, iNOS, COX-2 and RANKL induction at 3 and 9 dpi ($P < 0.05$) but not at 15 dpi ($P > 0.05$).

### 3.10. Concentrations of osteopontin and osteoprotegerin post PAI-1 treatment in S. aureus infected group

Serum (Fig. 10A) and synovial (Fig. 10B) levels of osteopontin (OPN) remained significantly higher in *S. aureus* infected group at 3, 9 dpi and 15 dpi ($P < 0.05$,). Treatment with PAI-1 inhibitor caused a marked decrement in OPN level in *S. aureus* infected arthritic mice

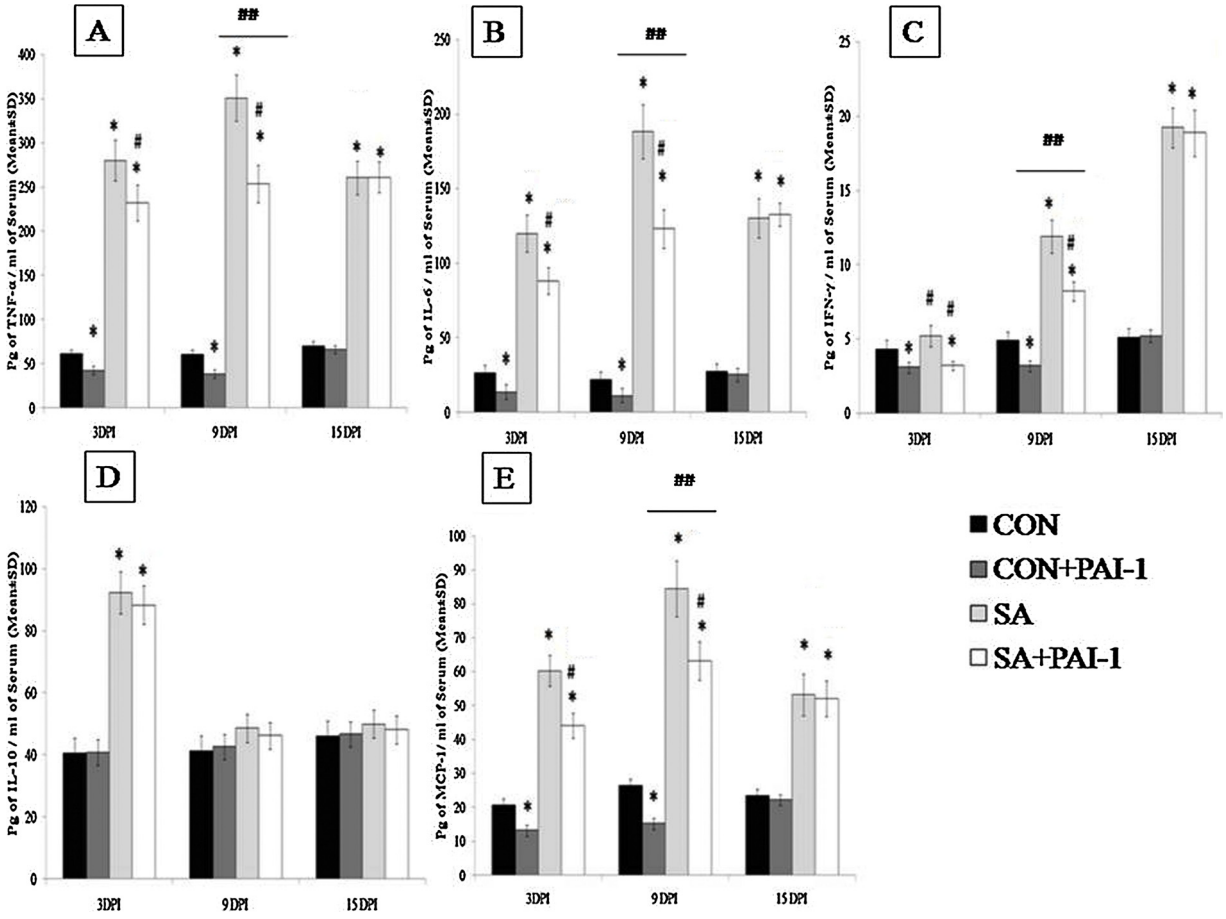


**Fig. 7.** Effect of administration PAI-1 after *S. aureus* infection on serum cytokine TNF-α (A), IL-6 (B), IFN-γ (C), IL-10 (D) and chemokine MCP-1 (E) at 3, 9 and 15 days post infection. Changes in serum cytokine TNF-α, IL-6, IFN-γ, IL-10 and chemokine MCP-1 were observed by ELISA method in CON, CON + PAI-1, SA and SA + PAI-1 groups during different days post infection. Values are expressed as mean ± SD from each group at different dpi. * indicates significant difference in comparison to CON ($P < 0.05$). # indicates significant difference in comparison to SA ($P < 0.05$). ## indicates significant difference in comparison to SA ($P < 0.01$).

(SA + PAI-1) both at 3 ($P < 0.05$) and 9 dpi ($P < 0.01$) but not at 15 dpi. Osteoprotegerin concentration in the serum (Fig. 10C) and the synovial tissue (Fig. 10D) was observed to be higher in *S. aureus* infected mice (SA). No significant alteration in OPG level was observed after PAI-1 administration at the earlier phase but the level of OPG decreased drastically at 15 dpi (Fig. 10C & Fig. 10D) ($P < 0.05$).

### 3.11. MPO activity in the synovial joint of arthritic mice post PAI-1 treatment

MPO enzyme activity is a direct marker of neutrophil accumulation. *S. aureus* infection caused higher MPO activity (Fig. 11A) in the synovial joints of *S. aureus* infected mice at 3 dpi ($P < 0.05$) but activity reduced at 9 and 15 dpi ($P > 0.05$) describing higher neutrophil accumulation at the earlier phase of infection. PAI-1 treatment led to significant decrement in MPO level in *S.aureus* infected arthritic mice (SA + PAI-1) at 3 dpi but not at 9 and 15 dpi.

### 3.12. Superoxide anion and nitric oxide concentration in the synovial joint after exogenous PAI-1 administration

*S. aureus* infection caused higher levels of superoxide anion (Fig. 11B) and nitric oxide (Fig. 11C) generation at 3, 9, 15 dpi compared to the control group ($P < 0.05$). A significant decline in the concentrations of super oxide anion and nitric oxide was found in the *S. aureus* infected groups treated with PAI-1 (SA + PAI-1) at 3 and 9 dpi ($P < 0.05$) but not at 15 dpi.

## 4. Discussion

*S.aureus* arthritis deserves special attention due to its serious clinical consequences and rapid resistance to available treatment options [47]. Plasmin activity remains remarkably higher during *S. aureus* arthritis than normal uninfected condition [14]. Several in vitro and in vivo studies have already shown that plasmin has the ability to stimulate the production of cytokines, reactive species, chemotaxis of monocytes/macrophages and release of other chemoattractant molecules which in a concerted manner contribute towards inflammatory destruction [48]. The conversion of plasminogen to plasmin is tightly regulated by the action of two plasminogen activators namely uPA and tPA. PAI-1, the major circulatory PAI binds irreversibly to both uPA and tPA thus inhibiting the generation of active plasmin [49]. uPA and tPA also play important roles in the induction of inflammation and tissue destruction during arthritis [50–52]. Therefore, excessive plasmin activity during *staphylococcal* arthritis might exacerbate the pathogenesis of the disease. Hence, in this study, we have tried to figure out the impact of exogenously administered PAI-1 on plasminogen/plasmin pathway that regulates the inflammatory cascade and arthritic destruction during *S. aureus* arthritis.

*S. aureus* infection was given intravenously through the mouse tail vein for the development of septic arthritis. This mouse model has been extensively used in our previously published works [29–31]. Followed by *S. aureus* infection, PAI-1 was intraperitoneally injected into the mice to inhibit both uPA and tPA, ultimately attenuating the process of plasmin generation. PAI-1 was chosen over other plasminogen activator

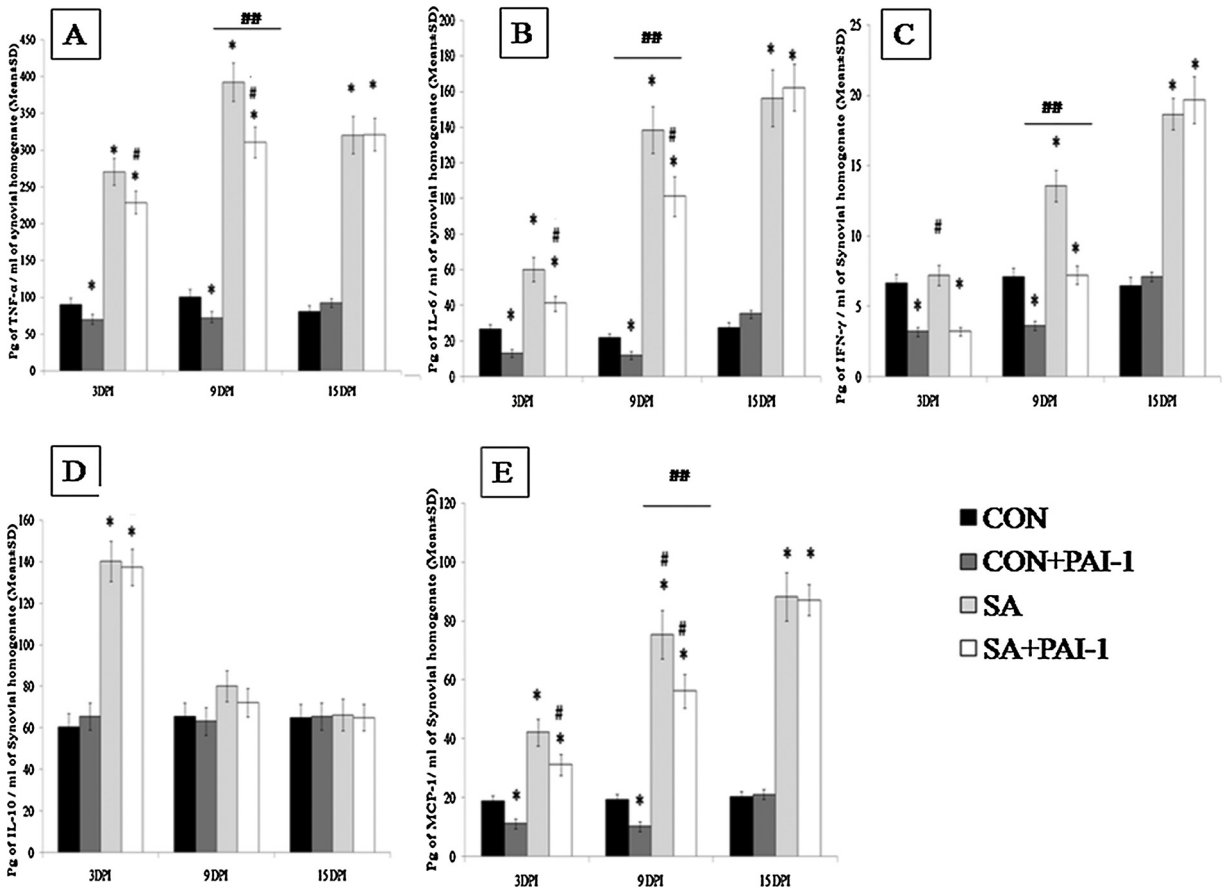


**Fig. 8.** Effect of administration PAI-1 after *S. aureus* infection on synovial cytokine TNF-α (A), IL-6 (B), IFN-γ (C), IL-10 (D) and chemokine MCP-1 (E) at 3, 9 and 15 days post infection: Changes in synovial tissue derived cytokine TNF-α, IL-6, IFN-γ, IL-10 and chemokine MCP-1 were observed by ELISA method in CON, CON + PAI-1, SA and SA + PAI-1 groups during different days post infection. Values are expressed as mean ± SD from each group at different dpi. * indicates significant difference in comparison to CON ($P < 0.05$). # indicates significant difference in comparison to SA ($P < 0.05$). ## indicates significant difference in comparison to SA ($P < 0.01$).

inhibitors such as tranexamic acid and aminocaproic acid because PAI-1 is naturally present inside the body whereas tranexamic acid and aminocaproic acid are synthetic compounds. Therefore, PAI-1 was more preferable and safer than tranexamic acid or aminocaproic acid. In addition to this, there are several reports stating ineffectiveness of tranexamic acid for the treatment of different forms of arthritis [53,54]. Progressive swelling of the knee joints and the paws was observed as a result of *S.aureus* infection during the whole arthritic episode. The results of the histological analysis clearly indicated *S.aureus* mediated severe bone and cartilage degradation, with rapid cellular infiltration, synovitis, tissue necrosis and bone destruction of the arthritic mice. In this context, treatment with PAI-1 in arthritic mice was found to have significantly decreased joint and paw swelling with less severe bone and cartilage destruction compared to the infected mice without PAI-1 treatment. However, the effect of PAI-1 treatment was not so prominent during the late phase of arthritis.

*S.aureus* infection led to higher uPA activity in the infected group whereas expression of tPA remained insignificant during the different days post infection. *S. aureus* has been reported to directly upregulate uPA expression [55]. Infection induced higher pro inflammatory cytokines can also stimulate uPA activity [56,57]. Thus, uPA remained prevalent throughout the course of arthritis. In addition to this, *S. aureus* infection seemed to have no direct effect on tPA expression during septic arthritis. Furthermore, there is no such established report justifying *S.aureus* induced tPA activation. Hence, the effect of PAI-1 administration was more prominent on uPA than tPA during *S. aureus* arthritis. Higher plasmin activity in the infected mice could also reflect *S. aureus* infection induced plasmin activation. These increased levels of plasmin and uPA might stimulate ECM destruction and initiation of inflammatory sequels in the affected joint [7]. These above findings were also reflected in our arthritis index and histopathological data. Therefore activation of plasmin and its activator uPA could have been severely impeded after continuous PAI-1 treatment. As a consequence, arthritis destruction was decreased remarkable in PAI-1 treated infected group. Indeed, plasmin and tPA activities were observed to be subnormal during late phase after continuous PAI-1 treatment. Subnormal plasmin activity might prevent removal of necrotic tissues. Low tPA activity due to PAI-1 inhibition, on the other hand, might attenuate the normal fibrinolysis process [2,58]. These accumulated necrotic tissues and fibrin particles in the affected joint could initiate a cascade of inflammatory reactions during the late phase. Although we did not determine fibrinolysis, however, according to histological analysis tissue necrosis was found to be higher at the late phase in the arthritic mice even after PAI-1 treatment.

Significant reduction in the bacterial load was assured after the treatment with PAI-1 in spleen and synovial tissues. The most possible explanation could be PAI-1 induced attenuation of MMP-2 expression. The involvement of plasminogen system in the processing and activation of MMP-2 has been already reported [59]. uPA itself triggers MMP-2 release from chondrocytes and synovial cells [60]. Moreover, PAI-1 could also inhibit MMP-2 activation directly [61]. Lack of integrity of the ECM as a result of MMP-2 mediated ECM destruction could increase the bacterial invasion in the tissue space with a subsequent higher bacterial load. Therefore, less MMP-2 expression might be one of the

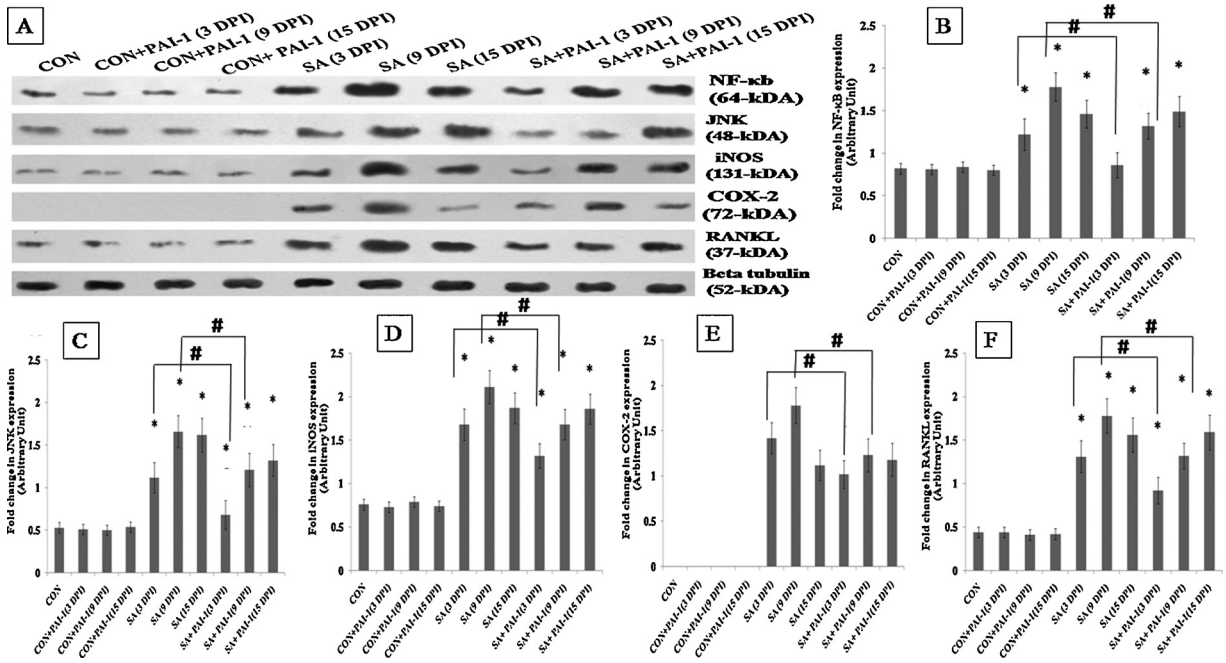


**Fig. 9.** Western blot analysis of expression of NF-κB, JNK, iNOS, COX-2 and RANKL in synovial joints: (A) Expressions of NF-κB, JNK, iNOS, COX-2 and RANKL were observed during different days post infection days in CON, CON + PAI-1, SA and SA + PAI-1 groups of mice. Densitometric data are expressed as arbitrary units normalized on the expression of the protein β –tubulin. Bar diagrams indicating expression of NF-κB (B), JNK (C), iNOS (D), COX-2 (E) and RANKL (F) in different groups of mice at 3, 9 and 15 dpi. * indicates significant difference in comparison to CON ($P < 0.05$). # indicates significant difference in comparison to SA ($P < 0.05$).

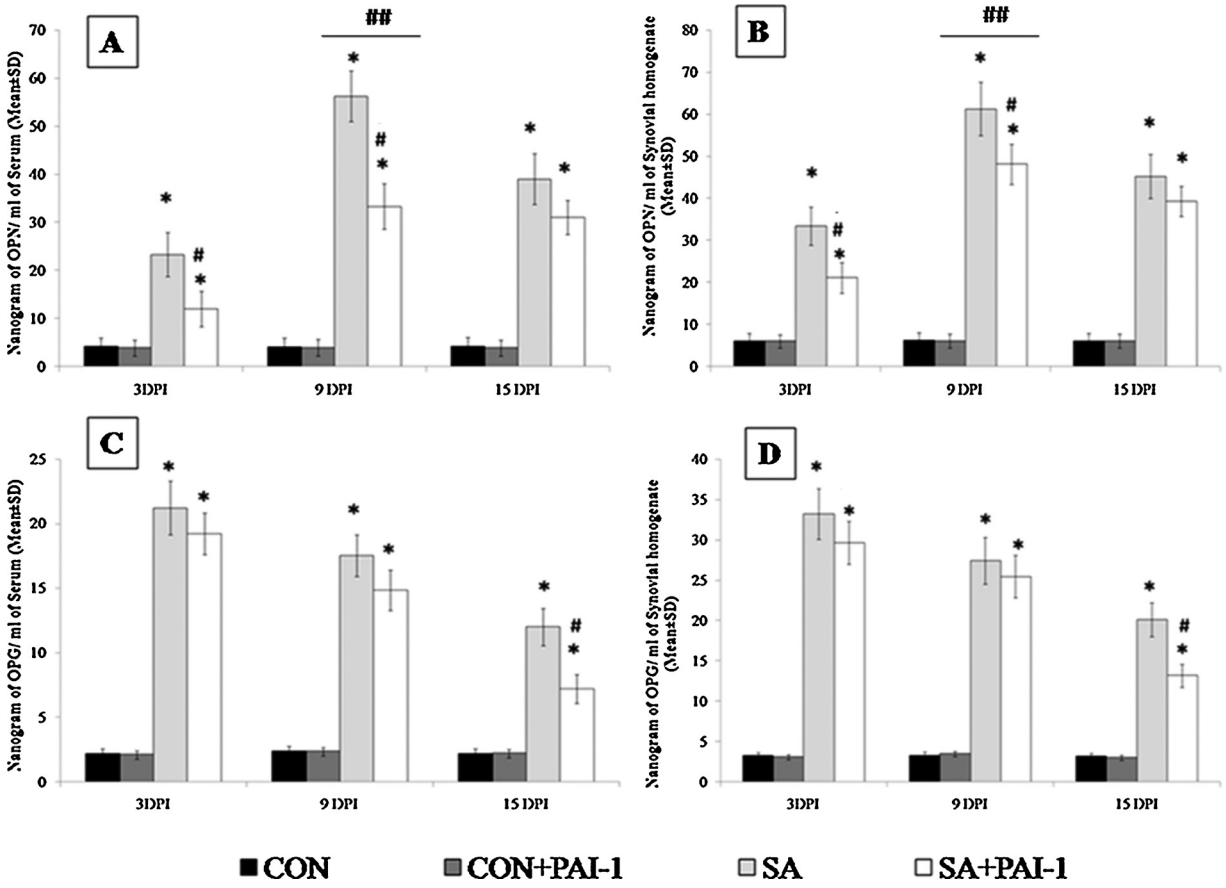


**Fig. 10.** Effect of administration PAI-1 after *S. aureus* infection on serum OPN (A), synovial OPN (B), serum OPG (C) and synovial OPG (D) at 3, 9 and 15 days post infection: Changes in concentrations of OPN and OPG were assessed both at serum and synovial tissue level during different days post infection. Values are expressed as mean ± SD from each group at different dpi. * indicates significant difference in comparison to CON ($P < 0.05$). # indicates significant difference in comparison to SA ($P < 0.05$). ## indicates significant difference in comparison to SA ($P < 0.01$).

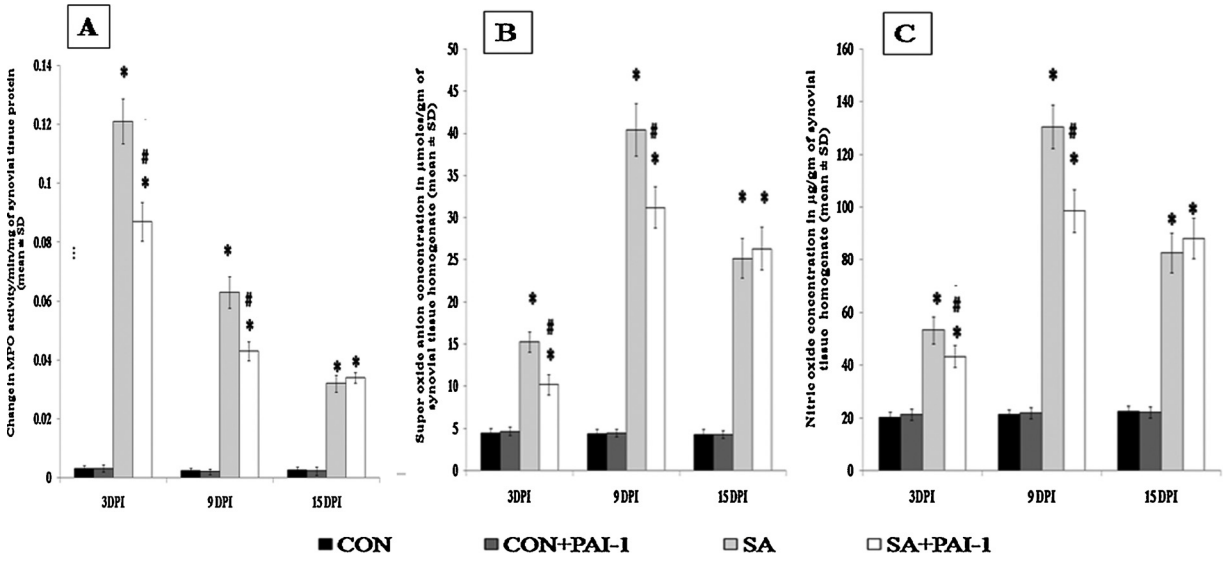


**Fig. 11.** Effect of PAI-1 treatment on synovial MPO activity, Super oxide and Nitric oxide concentrations at 3, 9 and 15 days post infection. Changes in MPO activity (A) and concentrations of Super oxide (B) and Nitric oxide (C) were determined at synovial tissue level during phases of arthritis. Values are expressed as mean ± SD from each group at different dpi. * indicates significant difference in comparison to CON ($P < 0.05$). # indicates significant difference in comparison to SA ($P < 0.05$).

important reasons behind reduction in bacterial count. In our previously published work, we also noticed a significant reduction in bacterial load after MMP-2 neutralization during septic arthritis [15].

Cytokines released from infiltrating macrophages and resident synovial cells in the affected joints play important roles during arthritis [62]. According to our ELISA report, higher levels of inflammatory cytokines like TNF-α, IL-6, IFN-γ were observed in *S. aureus* infected group when compared to the controls. Levels of these cytokines were reduced remarkably in infected group after PAI-1 treatment. Plasmin itself can act as a mediator of inflammation by inducing those aforesaid cytokines [63]. Hence, a steady decline in the plasmin concentration by PAI-1 treatment might be the reason behind decrement in the levels of these cytokines. Meanwhile, at 15 dpi low plasmin activity might enhance accumulation of necrotic tissue thereby stimulating generation of these inflammatory cytokines [13,64]. However, PAI-1 treatment did not affect anti inflammatory cytokine IL-10 level either in the serum or in the synovial tissue. Increased NF-κB and JNK signaling after *S. aureus* infection could also alleviate pro inflammatory cytokines production during septic arthritis [65]. Plasmin is known to activate both NF-κB and JNK pathways which in turn could augment these cytokines [23]. It could be positively correlated with PAI-1 administration and consequently inhibition of plasmin activity followed by suppression of NF-κB and JNK signaling.

MPO activity was found to be highest at 3dpi in arthritic mice denoting neutrophil accumulation at very early phase in response to inflammatory stimuli. MCP-1, an important chemokine associated with macrophage infiltration [66] was noticed to be higher mainly during mid and late phase suggesting activation and domination of invading macrophages compared to neutrophil during the later phase. Neutrophils or macrophages both release ROS as a host defense mechanism for bacterial killing [67,68]. However, excessive ROS production can lead to devastating consequences. Hence, generation of these reactive species should be controlled to prevent further destruction. Treatment with PAI-1 showed reduction in MPO activity and MCP-1 concentration remarkably. Plasmin in concert with other pro inflammatory cytokines is known to activate these phagocytes [69,70]. Hence, less plasmin activity after PAI-1 treatment should also be the reason behind marked decreased in superoxide anion and nitric oxide concentrations at the synovial tissue level. Plasmin also influences iNOS expression which in turn upregulates NO directly [71]. High inflammatory cytokines could also generate superoxide, iNOS and NO levels actively [72,73].

Bone-cartilage destruction remains one of the major concerns of septic arthritis. Formation of osteoclasts can directly influence bone resorption. Osteoclasts serve as the key players in the erosive and inflammatory events during the different forms of arthritis [74]. RANKL promotes osteoclastic bone resorption whereas OPG ameliorates RANK-RANKL interaction [75]. On the other hand, OPN stimulates matrix degradation as well as osteoclastogenesis [76]. TRAP staining confirmed low TRAP positive cells (supposed to be osteoclasts) in the infected group treated with PAI-1 when compared with infected only. However, at 15 dpi no significant change in TRAP positive cell count was found after PAI-1 treatment. Low RANKL and OPN in PAI-1 treated arthritic mice were another indication of the reduction of osteoclastogenesis during 3 and 9 dpi whereas OPG level decreased sharply at 15 dpi. PAI-1 has been reported to inhibit RANKL expression thus reducing bone destruction and progression of arthritis [77]. Lower plasmin and higher cytokines might abrogate OPG level resulting in enhanced oteoclastogenesis at the late phase of inflammatory arthritis. COX-2 expression was exacerbated after *S. aureus* infection but declined after PAI-1 treatment. This altered level of COX-2 might also influence bone resorption by stimulating RANKL and inhibiting OPG expressions [78]. Decrement in OPN level after PAI-1 treatment should also be due to the PAI-1 mediated inhibition of plasmin activity responsible for OPN activation.

In summary, our data demonstrated exogenously administered PAI-1 inhibited uPA and tPA mediated activation of plasmin with subsequent reduction in MMP-2, RANKL and OPN expressions during *S.aureus* induced septic arthritis. PAI-1 itself might directly inhibit MMP-2 and RANKL activation leading to a marked decrement in ECM destruction and bone degradation respectively. The decline in plasmin activity also caused a diminution in the levels of inflammatory cytokines such as TNF-α, IL-6 and IFN-γ, thereby, preventing inflammatory destruction. Despite having a beneficial role in suppressing the progression of arthritis during the earlier phase of arthritis, prolong PAI-1 treatment led to a subnormal plasmin activity which could oppose the benefits of PAI-1 treatment during the late phase. If the continuous PAI-1 administration had been stopped after 9 dpi, the results at 15 dpi could have been different. Hence further studies will be necessary to investigate more detailed and exact molecular mechanisms behind the therapeutic potential of PAI-1 in attenuating *S.aureus* induced septic

arthritis.

## Conflict of interest

The authors declare no financial conflicts of interest.

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