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Immunomodulatory effects of berberine on *Staphylococcus aureus*-induced septic arthritis through down-regulation of Th17 and Treg signaling pathways

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Objectives: The preseaant study was aimed to investigate the immunomodulatory effects of berberine on *Staphylococcus aureus*-induced septic arthritis through the downstream signaling mechanism of Th17 and Treg, in the control and prevention of disease progression of *Staphylococcus aureus* induced septic arthritis of blood, spleen and synovial joints. Methods: The study was conducted in mice induced with septic arthritis by *S. aureus* for 15 days. The infected mice were treated with berberine (50 or 100 or 200 mg/Kg) to evaluate the effects on the isolated cells of Th17 and Treg from synovial joints, blood and spleen against the septic arthritic induced mice followed by JNK, RANKL and NF-κB expressions in the lysates of Th17 and Tregs isolated cells. The evaluation of serum IL-21 and TGF-β levels was also conducted after 15 days post-infection in Th17 and Treg population. Results: Our findings showed that berberine exerted excellent inhibitory effects on the *S. aureus* (AS-789) strain for inducing sepsis-induced arthritis. The results from the *S. aureus* testing revealed that at concentrations below 640 µg/mL, the strain was more resistant to berberine, as it had an increased rate of growth. The assessment of *S. aureus* induced septic arthritis (joint swelling and arthritis index) substantial reduction in the joint swelling and arthritis index ($p<0.01$) in the berberine-treated groups. The percentage of Th17 cells with CD4 and RORγt; Treg cells with CD4, CD25 and FOXp3 in the synovial joints, blood and spleen was substantially declined in the drug-treated groups ($p<0.01$) as compared to the *S. aureus* infected mice. The TGF-β and IL-21 serum levels determinations in *S. aureus* induced septic arthritis revealed a substantial decrease in serum TGF-β levels ($p<0.01$) in drug-treated groups compared to the infected animals. The post hoc test revealed a substantial decrease in JNK, NF-κB and RANKL expressions in the lysates of Th17 and Treg isolated cells in the drug-treated animals ($p<0.01$) when compared to the *S. aureus*-infected cluster. Conclusion: Our findings demonstrated that a possible strategy for combating disease severity with berberine treatment in *Staphylococcus aureus* induced septic arthritis in mice, which targets the Th17 and Treg cells have driven the NF-κB/JNK-RANKL axis.

Keywords: Berberine, *S. aureus*, Treg cells, septic arthritis

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Abbreviations: AhR, Aryl hydrocarbon receptor; CMC, Carboxymethyl Cellulose; CYP1A1, Cytochrome P450, family 1, subfamily A; EDTA, Ethylenediaminetetraacetic acid; FACS, Fluorescence-activated cell sorting; FOXP3, Forkhead box P3 protein; IFN-γ, Interferon-gamma; IL-21, Interleukin-21; IκB, Inhibitor of nuclear factor kappa B; JNKc, Jun N-terminal Kinase; MAPK, Mitogen-activated protein kinase; MHB II, MuellerHinton broth II; MIC, Minimum inhibitory concentration; NF-κB, Nuclear factor kappa B; RANKL, Receptor activator of nuclear factor kappa-B ligand; RORγt, Transcription factors of the RAR-related orphan nuclear receptor (ROR) family; *S. aureus, Staphylococcus aureus*; TBST, Tris Buffered Saline with Tween; TGF-β, Transforming growth factor; Th2 cells, T helper T2 cells; Treg cells, Regulatory T cells; TSB, Tryptic soy broth

INTRODUCTION

Septic arthritis is typically characterized by a swollen and inflamed joint (Carpenter *et al.*, 2011). Septic arthritis remains a significant problem due to its high death and morbidity rates (Shirtliff & Mader, 2002). T cells and their subsets are critical for the integration of the innate and adaptive immune systems in numerous diseases in regulating disease severity. The T cells and cytokines perform a major function in the control and progression of *Staphylococcus aureus* septic arthritis. The Staphylococcal superantigen induces an influx of immune cells, particularly PMNs and macrophages, as well as significant T-cell (CD4+) infiltration (Abbas *et al.*, 1996) (Colavite-Machado *et al.*, 2013). The CD4 and T-helper cells differentiate into many sub-types after being exposed to antigens. Th17 cells are vital in the pathogenesis of many autoimmune and infective illnesses (Beringer *et al.*, 2014). These Th 17 cells grow independently of CD4 and T-helper cells and involve TGF-β and IL-6 produced by active immune cells to grow (Kimura & Kishimoto, 2010). The Th17 cells may produce pro-inflammatory cytokines such as IL-21, -17A and -17F (Lubberts, 2010).

The expression of RORγt and subsequent stabilization of Th17 cells exacerbate disease conditions following the IL-21R upregulation on the stimulated Th17 cells (Huber *et al.*, 2008). These Th17 cells are known to play a role in several arthritic models (Kugyelka *et al.*, 2016). The homotypic binding of IL-17A and its receptors cause osteoclast differentiation *via* the MAPK and NF-κB pathways (Ichiyama *et al.*, 2011; Ruan & Chen, 2012).

It also activates RANKL, which promotes osteoclast production in the synovial joint, resulting in bone loss (Chen *et al.*, 2016). The Th17 and T-regulatory cells formed from undistinguishable CD4 and T cells have distinct relationships with the existence of TGF-β (Kong *et al.*, 2012). The ability to tolerate endogenous proteins conferred by FoxP3 expression aids in the control of immunogenic inflammation (Fontenot *et al.*, 2003).

Many strategies have been used to exploit Tregs' immunosuppressive properties, including several arthritis models (Grant *et al.*, 2015). In many cases, an abnormal

Th17/Treg balance is linked to poor prognosis (Noack & Miossec, 2014). Inducible transcription factors control the progress, purpose of inflammatory and regulatory T cells. NF-κB, a central mediator of many inflammations, is activated *via* two main pathways: non-canonical and canonical (Sun, 2011; Vallabhapurapu & Karin, 2009; Oh & Ghosh, 2013). The NF-κB expression changes were linked to increased IL-6 production, a major mediator of Th17 differentiation (Chang *et al*., 2009). Also, ablating T cell-specific IKK reduced Th17 induction, demonstrating NF-regulatory B's role in modulating Th17 responses in autoimmune disorders like experimental allergic encephalomyelitis (Greve *et al*., 2007). The paradoxical role of NF-κB in the immune response is revealed by its ability to stimulate inflammatory genes while regulating Tregs. c-Rel is linked to both Th17 and Treg formation via its master transcription factor FoxP3 (Long *et al*., 2009; Ruan *et al*., 2009). NF-κB members such as IκB have been shown to influence Treg proliferation and function, which is disrupted in these conditions (Chang *et al*., 2012; Shi & Sun, 2015) T cell differentiation is closely linked to NF-κB and MAPK transcription factors. The MAPK genealogy consists of ERK, JNK, and p38 kinase. Inhibition of T cell proliferation, differentiation, and death by JNK family protein kinases (Dong *et al*., 2000)). RORγt expression increases in pathogenic Th17 cells with IL-6 (Sato *et al*., 2006; Kikuta *et al*., 2013).

These activated Th17 cells then produce IL-17, which increases JNK activation (Li *et al*., 2016; Li *et al*., 2016). The role of TGF-β in Treg generation from naive CD4+Th cells is not limited to the differentiation of Th17 cells. It assists in the adaptation of Treg differentiation *via* SMAD independent MAPK pathways, involving JNK/ERK, but without including p38 (Lu *et al*., 2010; Zhang, 2009). JNK inhibition impeded TGF-β mediated Treg proliferation in mice (Dominguez-Villar & Hafler, 2018; Arellano *et al*., 2016; Somayaji *et al*., 2008; Henningsson *et al*., 2010). TGF-β also activates JNK, increasing FoxP3 expression in Tregs, which has been linked to a reduction in disease severity. Th17 stimulates osteoclastogenesis *via* the RANK-RANKL axis, as well as NF-κB and JNK signaling pathways (Dominguez-Villar & Hafler, 2018; Lu *et al*., 2010). RANKL is an osteoclastogenic factor expressed by macrophages, synovial fibroblasts, CD4+T cells and osteoblasts (Arellano *et al*., 2016). Bone injury is mediated by RANK-RANKL interaction (Somayaji *et al*., 2008). Local IL-17 overexpression was corresponding along the higher RANKL expression in collagen-induced arthritis animals (Henningsson *et al*., 2010). Also, Th17-particular mediators like IL-6, IL-1, and TNF cause bone loss via a RANKL-dependent mechanism (Takayanagi, 2005). The RANKL/NFB axis causes significant bone injury and the development of inflammatory arthritis (Kikuta *et al*., 2013; Sato *et al*., 2006).

Natural compounds like Berberine have been studied for their pharmacological benefits, especially in the treatment of cancer, diabetes, atherosclerosis, and cardiovascular diseases (Cicero & Baggioni, 2016; Fatahian *et al*., 2020; Kumar *et al*., 2015; Ayati *et al*., 2017; Hu *et al*., 2011; Mortazavi *et al*., 2020). The antimicrobial outcome of berberine was tested against staphylococcus (coagulase-negative) strains by Zhou and others (Zhou *et al*., 2019). Berberine, which was studied by Chu and others (Chu *et al*., 2016) showed antibiotic activity against Methicillin-Resistant *S. aureus* (Moreland & Curtis, 2009). According to Guo and others (Guo *et al*., 2015), berberine was found to be efficient in preventing *S. aureus* growth in the biofilm cultures. These investigations, yet, used strains that were either non-clinical or not *S. aureus* strains.

Berberine therapy reduced collagen-stimulated splenocytes' producing inflammatory cytokines IFN-γ, IL-2, and IL-17 and increased joint stiffness in both collagen-induced arthritis (Wang *et al*., 2014; Yue *et al*., 2017) and Autoimmune and Autoinflammatory Arthritis (Wang *et al*., 2014) models. Berberine also lowered down bone loss, enhanced calcium restoration in AIA rats by inhibiting osteoclast proteolytic activity and lowering RANKL released in the joint area (Sujitha *et al*., 2020). Berberine's suppressive impact on bone erosion is subsequently confirmed, demonstrating inhibition of RANKL-related osteoclastogenic differentiation by suppressing NF-κB and Akt stimulation (Dinesh & Rasool, 2018; Hu *et al*., 2008). Many *in vitro* and *in vivo* investigations have shown that berberine has modulatory effects on Th17 and Treg cell function and proliferation in rheumatoid arthritis (Dinesh & Rasool, 2019). Berberine reported to drastically lower Th17 population blood levels and IL-17 serum levels in collagen-induced arthritis in rats. This effect is followed by a decrease in the synovium IL-17 expression and RORγt (Th17 transcription factor) in the spleen. Berberine therapy can drastically reduce Th17 cell development according to an *in vitro* study on naive T cells isolated from the spleen of autoimmune and autoinflammatory arthritis in rats, by down-regulating CD196 and RORγt. Consequently, berberine treated naive CD4 and T cells demonstrated development of CD4, CD25 and Foxp3 in Treg cells *via* the AhR/CYP1A1/Foxp3 axis (Dinesh & Rasool, 2019).

However, no research examining the immunomodulatory effects of berberine on staphylococcus aureus-induced septic arthritis including downregulation of Th17, Treg signaling pathways have been published so far. The berberine's immunomodulatory effects on staphylococcus aureus-induced septic arthritis were investigated through the downregulation of Th17 and Treg, in regulating the pathogenesis of *S. aureus*-induced septic arthritis. Additionally, the study examined the immunomodulatory effects of berberine on the various downregulating signaling pathways of Th17 and Treg cells obtained from arthritic mice's synovial joints, blood, and spleen.

MATERIALS AND METHODS

Animals

Adult male mice (Swiss albino; 25±2 g) were utilized in this experiment, and faunas were preserved in a controlled setting (temperature 25±3°C). The animal ethical committee permitted the use of animals (approval number: TCM-JL_12/45/2020). The experimental mice were given a standard mouse meal and free clean water. After 15 days, mice were segregated into control and *S. aureus* infected groups. The *S. aureus* affected category was further segregated into three subdivisions: Berberine (50 or 100 or 200 mg/Kg)). Each subgroup consisted of ten mice.

Culture of *Staphylococcus aureus*

Staphylococcus aureus was employed in this investigation, and it was procured from the Hospital in Beijing (AG-789; *S. aureus*-strain). The bacteria were kept in our lab and their antibiotic sensitivity was examined. Clinical isolates of this strain were utilized in previous animal models of arthritis to induce a non-lethal infection

temporarily (Dey & Bishayi, 2017). The bacterial isolates were incubated and washed using sterile PBS, and the inoculum was assessed using a spectrophotometer and the optical density was adjusted before infection (Optical Density 600=0.3=4×10^6 cells/ml for *S. aureus*) (Dey & Bishayi, 2020).

The minimum inhibitory concentration (MIC) of berberine

The MIC values of berberine against *S. aureus* strain (AG-789) were evaluated in triplicate using two-fold dilution series in MHB II (Mueller–Hinton broth II) as described before (Dey & Bishayi, 2017; Dey & Bishayi, 2020). The minimum inhibitory concentrations (MICs) were determined as the minimum concentrations at which no observable increase was seen after 24 hours. Berberine was added to a 96-well plate of TSB broth containing 1% glucose to make 100 µl as a final volume, and concentrations (5, 10, 20, 40, 80, 120, 160, 320, and 640 µg/ml) were prepared. The subsequent inoculation of cultures was carried out with 200 µl of *S. aureus* seeded culture (6×10^5 CFU/mL). The supernatant was entirely removed after incubation for 24 hours at 37°C, the wells were washed 3–4 times with phosphate-buffered saline (PBS) (pH 7.4). The biofilm was then stabilized for 20 minutes with 150 µl methanol before being dried at 50°C. The biofilm was then stained for 20 minutes with 220 µl of 0.2 percent crystal violet. PBS was used to rinse unbound crystal violet 3 times. Following drying, 300 µL of ethanol (90%) was introduced to the wells, and the plates were agitated to remove the stains from the biofilm for about 2 hours. The biofilm's absorbance was calculated three times at 600 nm. The bacterial growth control was performed using wells containing 1% DMSO, bacteria.

Bacterial growth control assessment

In 96-well plates, a suspension of 200 µL bacterial cultures (4×10^5 CFU/mL) in Tryptic soy broth (TSB) was introduced to 200 µL of serial dilutions of berberine (5, 10, 20, 40, 80, 120, 160, 320, and 640 µg/mL). Microplates were cultivated at room temperature for 3, 6, 12, and 24 hours, and bacterial growth was assessed using a microplate reader to measure the optical density at 620 nm wavelength. the wells comprising 1% DMSO and a bacterial inoculum represented as a control for bacterial growth.

Procedure for *S. aureus* induced arthritis

The methodology from our prior findings was used to generate septic arthritis in mice (1:2). The mice were given sterile phosphate (100 µL) buffered saline (PBS) comprising *S. aureus* inoculum through the tail vein. The control mice were given sterile PBS (100 µL). Each group was monitored regularly (4×10^7 CFU per mouse) for 15 days.

Treatment of drugs

After 15 days, the *S. aureus* diseased group was separated into three subgroups: Berberine (50 or 100 or 200 mg/Kg). Each subgroup consisted of ten mice. Gavage was used to administer the drug substances orally. Before oral administration, the drug substances were suspended in 10% CMC. Each of the three infected groups received medicines orally for 15 days after the infection period of fifteen days.

S. aureus induced septic arthritis evaluation

A Vernier calliper was used every day to check the oedema of the mice's paws, ankle joints, and synovial knee joints. The severity of joint swelling was represented by these average values (Bettelli *et al.*, 2008) Induction (%) of arthritis in each group of treated and untreated mice was computed as: (Mean diameter (synovial knee joint swelling) in *S. aureus* infected mice on 15th day – Mean diameter (synovial knee joint swelling) in control mice on 15th day)/Mean diameter (synovial knee joint swelling) in control mice on 15th day) multiplied by 100. The significant differences noticed in the joint swelling reduction between the groups were evaluated statistically. The clinical severity of paw swelling due to arthritis was rated on a scale from 0 to 3 according to the modifications observed in swelling and erythema for each paw (0 – no modification; 1 – mild erythema and swelling; 2 – moderate erythema and swelling; 3 – marked erythema, swelling, and ankylosis). To calculate the arthritis index, the overall score was divided by the no. of fauna's put-to-use under each category (Puliti *et al.*, 2002).

Collection of specimens

The control, *S. aureus* infected, and drug-treated mice were anaesthetized with inhalation anaesthetics (ether) on the scheduled days after infection. General anaesthesia with anaesthetic ether was used for euthanasia, which was followed by tissue removal for further processing. A cardiac puncture was used to collect blood (1 mL), following the spleens and synovial tissues isolation.

Lymphocyte isolation from blood

Blood was extracted after euthanasia to mice through heart puncture and accumulated in precoated heparin micro-centrifuge tubes (100 µL). The blood collected was then diluted with the same amount of phosphate buffer saline (1:2) and centrifuged at 1000×*g* for 30 minutes. The lymphocyte film was isolated and then rinsed with phosphate buffer saline after centrifugation (Mizobe *et al.*, 1982). Isolated lymphocytes from each mouse were accumulated in a centrifuge (15 mL) tube, revamped into microcentrifuge tubes containing fluorochrome-conjugated antibodies for fluorescence-activated cell sorting. Experiments were done in triplicate.

Spleen lymphocytes isolation

Spleens were homogenized in an ice bath with Alsever's solution for the collection of splenic lymphocytes. Isolation of lymphocytes was achieved using single-cell suspension *via* repeatedly aspirating tissue above lymphocyte separation media in a 3:2 ratio and afterwards centrifuged at 1000×*g* for 30 minutes. To remove adherent cells, isolated cells were cultured for one hour at room temperature with 5% CO_2 in an incubator before being washed with RPMI-1640 medium. Lymphocytes were categorized as non-cells (Johansen *et al.*, 1974). Lymphocytes were extracted from the spleens of ten mice and pooled in a centrifuge (15 mL) tube before being reallocated in 3 microcentrifuge tubes containing fluorochrome antibodies for fluorescence-activated cell sorting (FACS).

Synovial joint lymphocyte isolation

The synovial joints were isolated from the hind paws of the infected and control mice. The isolated joints were treated individually with 4 mg/mL of collagenase-

D in phosphate buffer saline for 40 minutes at room temperature. The digestion was then halted by adding EDTA (10 mM) and incubated for another 15 minutes (Rampersad *et al.*, 2011). The transparent cell solution was then placed as layers onto the medium for lymphocyte separation, centrifuged at 4000 rpm for 20 minutes. The lymphocyte layers were later collected as described before. Lymphocytes were extracted separately from both synovial joints of ten mice for each experimental group, were then gathered in 15 mL capacity centrifuge tubes and transferred into the micro-centrifuge tubes accompanied by the conjugation of fluorochrome antibodies for fluorescence-activated cell sorting.

Antigen staining to assess the ratio of Th17/Treg cells

Isolated lymphocytes were obtained and collected in micro-centrifuge tubes from the synovial joints, blood and spleen of the control, infected and drug-treated mice for flow cytometric evaluation. Discolouring of several intracellular and extracellular markers was carried out for the separation of Th17 and Tregs as previously described (Dey & Bishayi, 2017, 2020; Rampersad *et al.*, 2011). Later re-suspension of cells was done in 200 µL FACS buffer and stained with the following surface markers: CD4 antibody conjugated with PE-Cy5 (1 µg per 4×10^6 cells) and CD25 antibody conjugated with FITC were added (15 µg per 10^7 cells). Cells were washed and fixed with 3% formaldehyde after 20 minutes at 4°C incubation. We next reconstituted the cells and added intracellular markers to them in a 0.5 percent saponin buffer before resuspending them again: PE-conjugated RORγt antibody (0.3 µg per 10^6 cells); APC conjugated FoxP3 (0.135 µg per 10^6 cells) according to maker's protocol. Cells were rinsed and re-suspended in FACS buffer after 20 minutes. Lymphocytes extracted from control and S.auerus infected mice's synovial joints were treated with surface markers labelled with fluorochrome, accompanied by permeabilization of cells with intracellular labelling. Before acquiring samples with multiple stains, we determined the fluorochrome compensation values using unstained and single fluorochrome stained samples. Plots were generated with CD4+ T cells, and 8000-9000 events were calculated to estimate the fraction of Th17 cells and Treg cells (Wilde *et al.*, 2012).

Preparation of samples for cytokine analysis

The blood samples were coagulated at 4°C before centrifugation at 500×g for about 10 minutes to determine serum cytokine levels. The serum supernatant was obtained, kept at –80°C, and utilized for the experiment. Before this analysis, all samples from individual groups were standardized for protein content using the Bradford method, and the levels of IL-21 and TGF-β were assessed using an ELISA kit (Majhi *et al.*, 2014; Dey & Bishayi, 2020) following the recommendations of the manufacturer's protocol. The least determining IL-21 and TGF-β values were <7 pg/ml and 0.61 ng/L respectively. The intra- and inter-assay reproducibility of IL-21 cytokine kits: CV <12%: CV <15%. The intra- and inter-assay reproducibility of TGF-β cytokine kits: CV <10%: CV <12%.

Western blot analysis

Isolated lymphocytes from synovial joints, blood, and spleen were analyzed using the western blot procedure. Then, using intracellular (RORγt and FoxP3) and surface markers (CD4 and CD25) for Th17 and Treg cells, fluorescence-activated cell sorting (FACS) was used to sort Th17 and Treg cells. After lysing, the Th17 and Treg cells in RIPA buffer supplemented with Nonidet P-40, the cell lysates were denatured with an equivalent protein concentration for 10 minutes at 80°C, re-determined using 10% SDS PAGE, and transferred onto a nitrocellulose membrane. The blocking buffers containing bovine serum albumin (BSA, 5%) used were TBST (15 mM Tris-hydrochloride, pH 7.2), sodium chloride (100 mM), Tween-20 (0.2%) at 4°C for 3 hours. Membranes were then rinsed thrice with TBST, allowed to conjugate in TBST supplemented BSA (2%) overnight with primary antibody (RANKL), NF-κB and JNK kinase at 4°C. The blots were washed three times with TBST at 4°C with HRP conjugated secondary antibodies for about 3 hours. The chemiluminescent-substrate was used to generate the final signal. Each blot was then quantified with Bio-Rad Software.

Statistical analysis

Each group's outcomes were expressed as mean ±S.D. (n=10). A one-way study of variance was utilized to assess significant variations between clusters (ANOVA). One-way ANOVA was used to determine whether there were significant differences in the means among the groups, following Dunnett's *t*-test with $p<0.05$ as statistically significant for the outcomes.

RESULTS

Berberine exerted excellent inhibitory effects on *S. aureus* (AS-789) strain for inducing sepsis-induced arthritis

There was a range of MIC values for berberine from 80 to 640 g/ml. The MIC value for the highest strain was 640 g/ml, while the minimum was 80 g/ml. There was no substantial growth of the tested *S. aureus* after 2 hours of incubation (Fig. 1a and b). When compared to the control group after six hours of incubation, the trial strain depicted a considerable reduction in the total count of bacteria (as demonstrated *via* variations in OD value) (1 percent DMSO, Fig. 1c). A substantial decline in the proportion of bacteria, with total growth inhibition of the strain, was noted after incubation for 12 hours with a range of berberine concentrations starting with 40 and at maximum 640 µg/mL (Fig. 1d). Despite some growth, the number of microbes in the range of berberine concentrations from 40 to 640 g/mL decreased significantly after 24 hours. The bacteria stopped growing completely when the concentrations of 320 and 640 µg/mL were reached, and the OD value remained the same (Fig. 1e). The results from the *S. aureus* testing revealed that at concentrations below 640 µg/mL, the strain was more resistant to berberine, as it had an increased rate of growth.

Assessment of *Staphylococcus aureus*-induced septic arthritis (joint swelling and arthritis index)

Induction of arthritis was assessed in control, infected and drug-treated mice from the swelling of the synovial joints. The research discovered that infected mice had greater joint size, which began to expand over time, starting from day 0 to day 15. Infected mice showed the

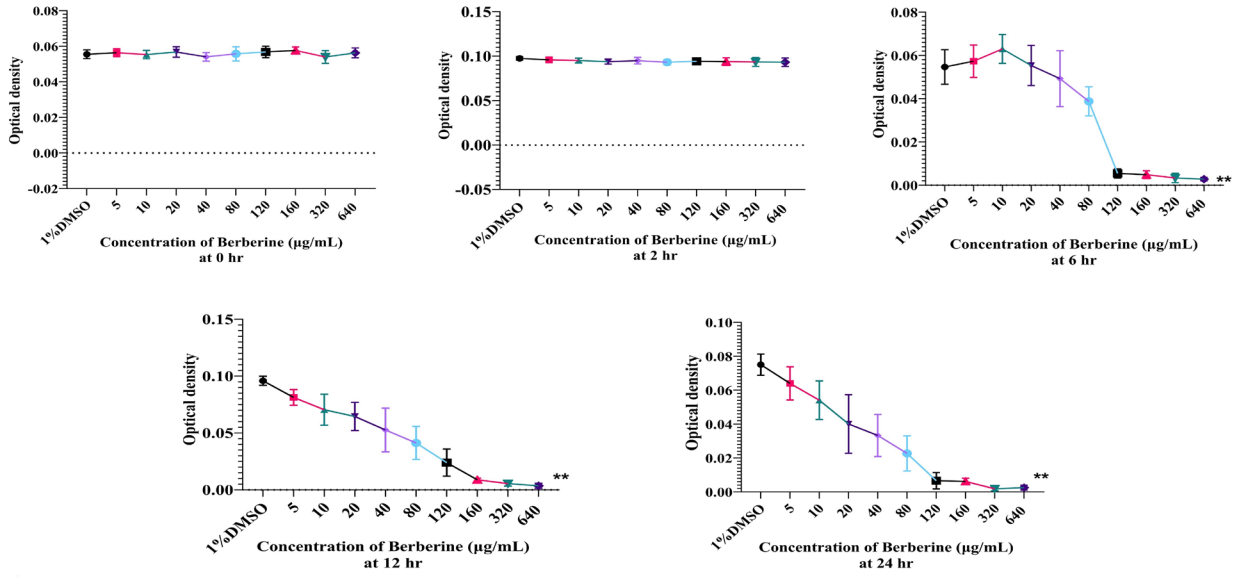

Figure 1. Growth kinetics of clinical S. aureus in the presence of different berberine concentrations after incubation of 0 h (a); 2 h (b); 6 h (c); 12 h (d) and 24 h (e)

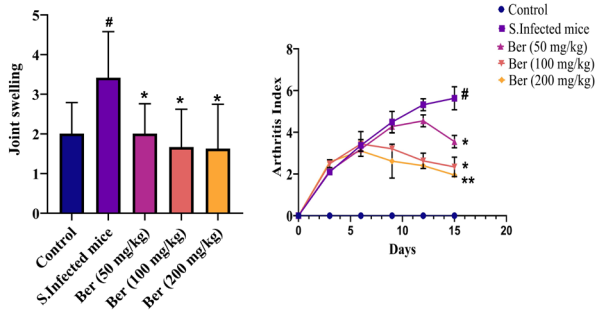

Figure 2. Experimental evaluation of joint swelling and arthritis index in *S. aureus* infection induced arthritis at different berberine (Ber) at 50, 100 and 200 mg/kg.
Values were expressed as mean ±S.D. of six mice per group ($p<0.05$). #indicates significant difference in comparison to control group. *$p<0.05$ and **$p<0.01$ indicates significant difference in comparison to infected groups.

greatest increase in joint oedema and arthritis induction as compared to control mice on day 15. The one-way ANOVA study of the berberine (50 or 100 or 200 mg/Kg) treatments for 15 days revealed significant differences across groups. The post hoc test revealed that the drug-treated groups had significantly less joint swelling ($p<0.01$) and arthritis induction ($p<0.01$) than the infected cluster. Infected animals showed a significant increase in the arthritis index since day-4 post-infection considered alongside control animals. A gradual increase in the arthritis index was observed since day 4, and the highest arthritis index was observed on 15-day (Fig. 2).

The arthritis index did not change significantly in the control mice. The 15-day therapy with berberine (50 or 100 or 200 mg/Kg) revealed significant differences across groups, according to one-way ANOVA analysis. The post hoc analysis revealed a substantial reduction in the arthritis index ($p<0.01$) in the drug-treated groups.

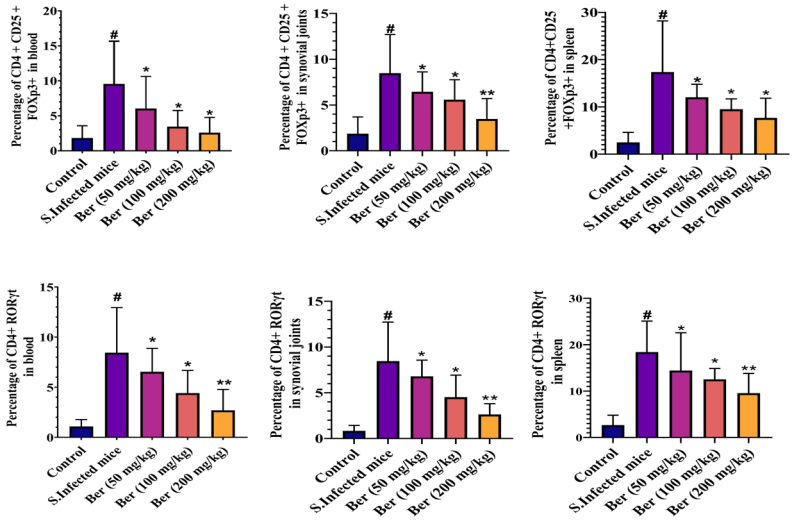

Figure 3. Percentage of FACS Sorted CD4+RORγt+Th17 cells and CD4+CD25+FoxP3+Treg cells in Blood, Spleen and Synovium in control, *S. aureus* infected and berberine treated mice.
Percentage of lymphocytes were expressed as mean ± S.D. of six mice per group ($p<0.05$). #indicates significant difference in comparison to control group. *$p<0.05$ and **$p<0.01$ indicates significant difference in comparison to infected groups.

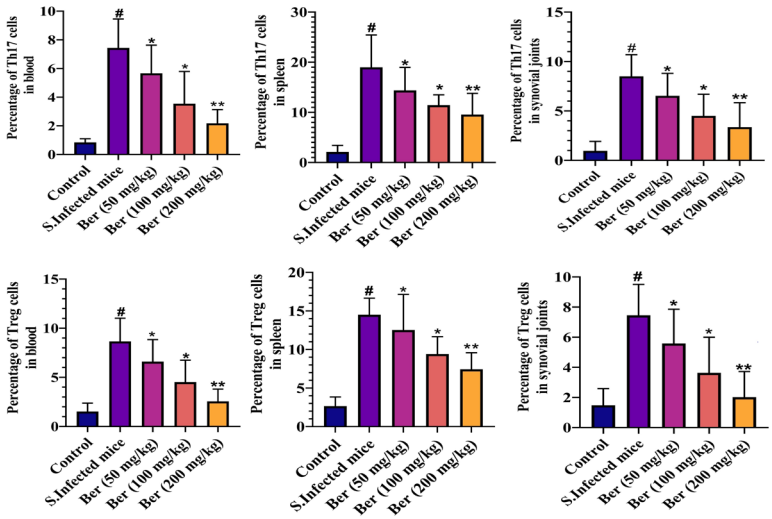

Figure 4. Graphical representation of percentage of Th17 and Treg cells isolated by FACS from Blood, Spleen and Synovial joints in Control, *S. aureus* infected and Berberine treated mice and collective analyses of Th17 cells (expressed as % cells) sorted from whole lymphocyte population obtained from blood, spleen and synovial joints.
Values were expressed as mean ± S.D. of six mice per group ($p<0.05$). #indicates significant difference in comparison to control group. *$p<0.05$ and **$p<0.01$ indicates significant difference in comparison to infected groups.

Analysis of flow cytometry to determine the percentage of Th17 cells with CD4 and RORγt and Treg cells with CD4, CD25 plus FOXp3 in the synovial joints, spleen and blood

The control, *S. aureus*-infected and drug-treated mice septic were given different extracellular and intracellular markers to extract Treg and Th17 cells from the synovial joints, spleen and blood to assess the modification in Th17 and Treg cells (Fig. 3). The clarity of insulated Th17 and Treg cells post arranging was higher than 97 percent in each sample. The percentage of Th17 cells in blood was considerably higher in the diseased cluster relative to the uninfected group. The amount of Th17 cells in the infected group increased significantly ($p<0.01$) after 15 days post-infection (Fig. 3). The 15-day therapy with berberine (50 or 100 or 200 mg/Kg) revealed significant differences among groups, according to a one-way ANOVA analysis. The post hoc analysis reveals a substantial decline in the percentage of Th17 cells in drug-treated groups ($p<0.01$) as compared to the *S. aureus* infected mice.

The *S. aureus* infected group (CD4+, CD25+, FoxP3+, Treg cells) had more of a Treg population than the control group due to a considerable rise in the number of infected Treg cells in the infected group. The post-infection Treg population was observed to grow sharply after 15 days (Fig. 3). The one-way ANOVA analysis revealed significant differences between groups after 15 days of therapy with berberine (50 or 100 or 200 mg/Kg). The post hoc test revealed a substantial decrease ($p<0.01$) in the number of CD4+; CD25+; FoxP3+ and Treg cells in the drug-treated groups compared to the infected group.

The population of Th-17 was enhanced among the sick animals after fifteen days of post-illness when compared to control animals in the case of splenic T lymphocytes (Fig. 4). The Treg population of the spleen in infected animals had a considerably higher population than that of the control group. The one-way ANOVA study of the berberine (50 or 100 or 200 mg/Kg) treatments for 15 days revealed notable differences among groups. The post hoc test revealed a substantial decrease in the number of splenic T lymphocytes (Th17) and splenic Treg population ($p<0.01$) in drug-treated groups compared to infected animals.

The Th17 cells were observed to be more prevalent in infected synovial joints than in the control group. Th17 cells were most abundant during 15 days of post-infection (Fig. 4). The percentage of Treg cells was also dramatically enhanced after 15 days post-infection (Fig. 5). The 15-day treatment with berberine (50 or 100 or 200 mg/Kg) revealed important changes between groups when studied using one-way ANO-

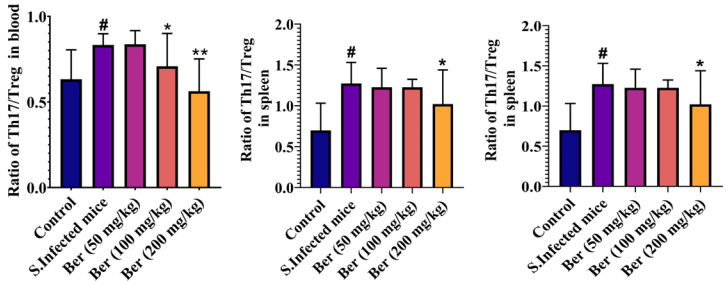

Figure 5. The Th17/Treg cells ratio in Blood, Spleen and Synovium in control, *S. aureus* infected and berberine treated mice.
#indicates significant difference in comparison to control group. *$p<0.05$ and **$p<0.01$ indicates significant difference in comparison to infected groups.

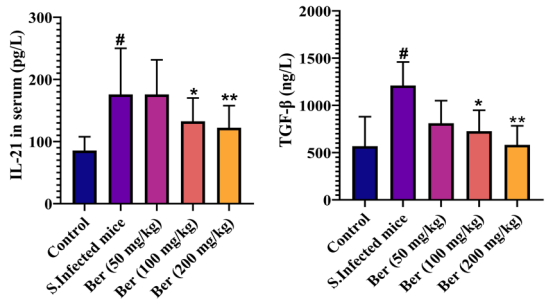

Figure 6. Serum levels of IL-21 and TGF-β in control, *S.aureus* infected and Berberine treated mice.
Values were expressed as mean ± S.D. of six mice per group ($p<0.05$). #indicates significant difference in comparison to control group. *$p<0.05$ and **$p<0.01$ indicates significant difference in comparison to infected groups.

VA. The after hoc analysis revealed a substantial decline in the number of splenic T lymphocytes (Th17) and splenic Tregs in synovial joints ($p<0.01$) in the drug-treated groups equated to the infected cluster.

A fraction of FoxP3 and CD4 of the Treg cells were found in the lymphocytes obtained from synovial joints spleen and blood. The Th17 and Treg ratios were separated during Treg cell gating from lymphocytes indicated a substantial boost in the Th17/Treg ratio relative to the blood and tissues of control mice (Fig. 5). The one-way ANOVA analysis revealed significant differences between groups after 15 days of therapy with berberine (50 or 100 or 200 mg/Kg). The post hoc analysis reveals a substantial drop in the Th17/Treg ratio in the blood and tissues of drug-treated groups ($p<0.01$) as compared to the infected group (Fig. 4).

Serum TGF-β and IL-21 determinations in *S. aureus* induced septic arthritis

To measure the amounts of TGF-β and IL-21, the serum of the uninfected, infected, and drug-treated mice were collected. The serum level of IL-21 was considerably elevated in the infected set as equated to the uninfected control cluster. The increase in IL-21 levels occurred within 15 days following infection in the infected group (Fig. 6). The one-way ANOVA study of the berberine (50 or 100 or 200 mg/Kg) treatments for 15 days revealed significant differences between groups. The post hoc test revealed that the drug-treated groups had significantly lower serum levels of IL-21 ($p<0.01$) than the infected group. The infected group had considerably higher levels of TGF-β than the control group (Fig. 6). The 15-day therapy with berberine (50 or 100 or 200 mg/Kg) revealed significant differences across groups, according to a one-way ANOVA analysis. The post hoc test revealed a substantial decrease in serum TGF-β levels ($p<0.01$) in drug-treated groups compared to the infected animals.

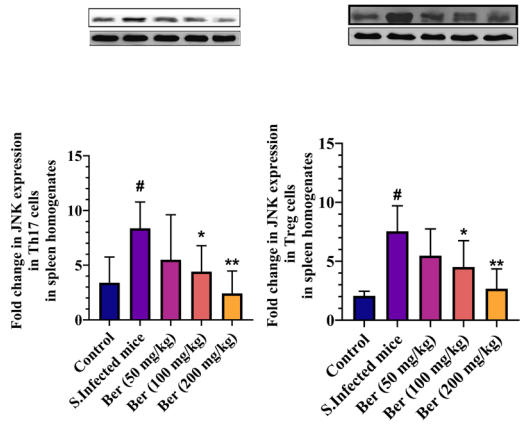

Figure 8. Western blot expression of JNK from lysate of Th17 and Treg cells in spleen homogenates.
Schematic expression of JNK expression in the FACS isolated Th17 and Treg cells from spleen homogenates. Respective fold-changes in expression are shown in graph. All membranes were stained with anti-beta-tubulin antibody to assure equal protein loading in the gels occurred. Expressions of JNK were measured in terms of fold-change compared to control. Experiments were repeated three times with similar results ($p<0.05$). Values were expressed as means ± S.D. #indicates significant difference in comparison to control group. *$p<0.05$ and **$p<0.01$ indicates significant difference in comparison to infected groups.

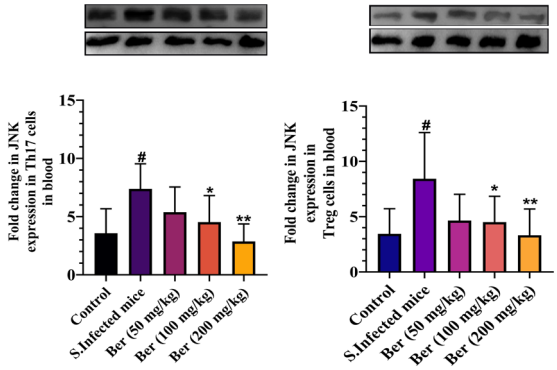

Figure 7. Western blot expression of JNK from lysate of Th17 and Treg cells in blood.
Schematic expression of JNK expression in the FACS isolated Th17 and Treg cells from blood. Respective fold-changes in expression are shown in graph. All membranes were stained with anti-beta-tubulin antibody to assure equal protein loading in the gels occurred. Expressions of JNK were measured in terms of fold-change compared to control. Experiments were repeated three times with similar results ($p<0.05$). Values were expressed as means ± S.D. #indicates significant difference in comparison to control group. *$p<0.05$ and **$p<0.01$ indicates significant difference in comparison to infected groups.

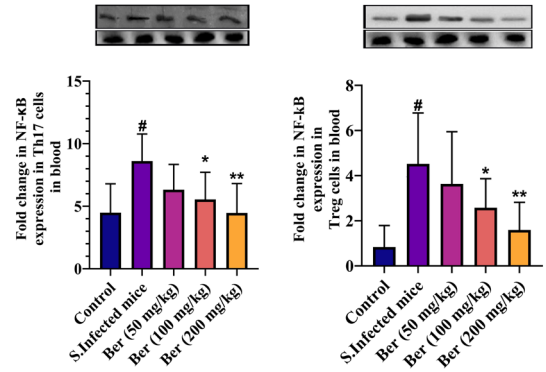

Figure 9. Western blot expression of NF-κB from lysate of Th17 and Treg cells in blood. Schematic expression of NF-κB expression in the FACS isolated Th17 and Treg cells from blood.
Respective fold-changes in expression are shown in graph. All membranes were stained with anti-beta-tubulin antibody to assure equal protein loading in the gels occurred. Expressions of NF-κB were measured in terms of fold-change compared to control. Experiments were repeated three times with similar results ($p<0.05$). Values were expressed as means ± S.D. #indicates significant difference in comparison to control group. *$p<0.05$ and **$p<0.01$ indicates significant difference in comparison to infected groups.

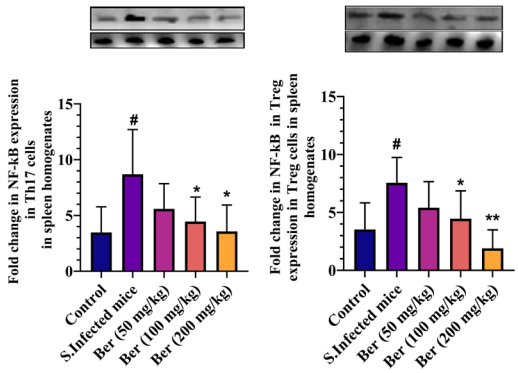

Figure 10. Western blot expression of NF-κB from lysate of Th17 and Treg cells in spleen homogenates.
Schematic expression of NF-κB expression in the FACS isolated Th17 and Treg cells from spleen homogenates. Respective fold-changes in expression are shown in graph. All membranes were stained with anti-beta-tubulin antibody to assure equal protein loading in the gels occurred. Expressions of NF-κB were measured in terms of fold-change compared to control. Experiments were repeated three times with similar results ($p<0.05$). Values were expressed as means ± S.D. #indicates significant difference in comparison to control group. *$p<0.05$ and **$p<0.01$ indicates significant difference in comparison to infected groups.

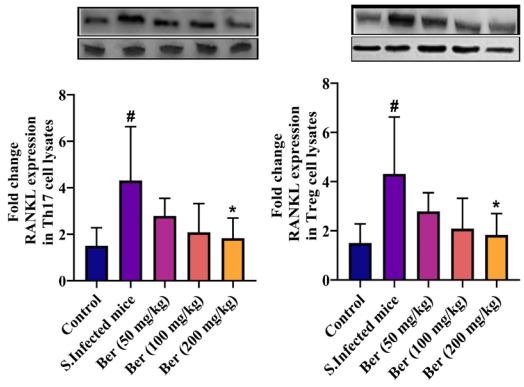

Figure 11. Western blot expression of RANKL from lysate of Th17 and Treg cells in synovial joints.
Schematic expression of RANKL expression in the FACS isolated Th17 and Treg cells from synovial joints. Respective fold-changes in expression are shown in graph. All membranes were stained with anti-beta-tubulin antibody to assure equal protein loading in the gels occurred. Expressions of RANKL were measured in terms of fold-change compared to control. Experiments were repeated three times with similar results ($p<0.05$). Values were expressed as means ± S.D. #indicates significant difference in comparison to control group. *$p<0.05$ and **$p<0.01$ indicates significant difference in comparison to infected groups.

Western blot analysis

In the synovial joints blood and spleen of *S. aureus* infected mice, JNK expression was considerably more in the isolated Th17 cells lysates than in the uninfected group ($p<0.05$) (Fig 7). The 15-day therapy with berberine (50 or 100 or 200 mg/Kg) revealed significant differences among groups, according to a one-way ANOVA analysis. The post hoc test revealed a substantial decrease in the JNK expression of the Th17 isolated cell lysates from drug-treated mice ($p<0.01$) when compared to the *S. aureus*-infected group.

The isolated Treg cells expressed JNK (Fig. 7) at a considerably greater level in the diseased group relative to the control group ($p<0.05$). The one-way ANOVA study of the berberine (50 or 100 or 200 mg/Kg) treatments for 15 days revealed significant differences among groups. The post hoc test revealed a substantial decrease in JNK expression in the isolated Treg cells lysates the drug-treated animals ($p<0.01$) when equaled to the *S. aureus*-infected cluster.

The NF-ϰB expression was significantly elevated in Th17 cells from the *S. aureus* group in the blood, spleen and synovial joint when compared to the control group ($p<0.05$) (Fig. 8 and 9). The one-way ANOVA analysis revealed significant differences between groups after 15 days of therapy with berberine (50 or 100 or 200 mg/Kg). In the post hoc test, the expression of NF-ϰB in Th17 cells of drug-treated mice was considerably lower ($p<0.01$) than in the *S. aureus*-infected cluster.

Isolated regulatory T cells (Tregs) from the synovial joints, blood and spleen were also found to express nuclear factor-ϰB (Fig. 9 and 10). The one-way ANOVA study of the berberine (50 or 100 or 200 mg/Kg) treatments for 15 days revealed significant differences among groups. The post hoc test revealed a substantial decrease in NF-ϰB expression in isolated Treg cells of the drug-treated animals ($p<0.01$) relative to the *S. aureus* infected set of animals.

To find out how bone resorption occurs, we identified the expression of the RANKL in Th17, Treg and CD4+ cells from synovial joints. The *S. aureus* group showed the Th17 cells significantly higher RANKL expression as compared to the control group ($p<0.05$) (Fig. 11). The 15-day therapy with berberine (50 or 100 or 200 mg/Kg) revealed significant differences among groups, according to a one-way ANOVA analysis. The post hoc test revealed a substantial decrease in the expression of RANKL in Th17, Treg and CD4+ cells from synovial joints of drug-treated animals ($p<0.01$) relative to the *S. aureus*-infected cluster.

DISCUSSION

Because berberine has shown efficacy against numerous microorganisms, such as *S. aureus* (Chu *et al.*, 2016; Imenshahidi & Hosseinzadeh, 2016), it is worth testing to see if the drug can be effective in treating *S. aureus*-induced septic arthritis. *S. aureus* was first tested for its sensitivity to berberine in our study. According to the findings, the maximum MIC value in *S. aureus* (AS-789) was found to be 640 μg/ml, and the minimum was found to be 40 μg/ml. When *S. aureus* (AS-789) was exposed to berberine, substantial inhibition of growth was observed. The growth evaluation tests indicated that *S. aureus* was sensitive to berberine, which was consistent with the results of the MIC test. In light of these considerations, we propose that the reason for this might be that berberine does not exhibit antibacterial activities contrary to staphylococci by active carnage, but preferably by hampering their growth.

The pathogenic infection triggers an excessive immune response that releases significant quantities of inflammatory cytokines even after the bacteria have been eradicated using modern antibiotic therapies. This leads to joint degeneration and bone resorption (Carpenter *et al.*, 2011). While many additional factors contribute to inflammation of different tissues, staphylococcal antigenicity and bacterial cell-wall components such as toxins, that are highly effective at triggering host immune activation (van Langevelde *et al.*, 1998). Despite advances in medical therapy, septic arthritis continues to be a source of concern because no effective medication has been developed to offset the bone injury caused by the enormous inflammatory response, that finally results in bone disintegration and deterioration of bone function. The development of an effective therapy that can modulate the increased immunological stress and bone degrada-

tion allied with *S. aureus*-induced septic arthritis is critical. The ability of *S. aureus* to generate and develop septic arthritis necessitates a full understanding of the underlying processes. Our study findings report that joint swelling and arthritis induction greatly improved after treatments with berberine in septic arthritis induced by *S. aureus* in mice. The immunological changes generated by berberine therapies after illness induction are supported by our findings.

T-cell activation and staphylococcal entrance are well-established correlations. CD4+ T helper cells are activated as soon as the pathogen enters the body; this creates an immune system that is composed of the innate and adaptive immune systems (Abbas *et al*., 1996). Th17 cells, a subset of these CD4+ T cells, may have a part in the generation of inflammation in a variety of disorders, including several arthritis models (Zambrano-Zaragoza *et al*., 2014). Berberine (50, 100 and 200 mg/Kg) therapy reduced septic arthritis inflammation by reducing the amount of CD4+ T cells and Th17 cells found in the synovial joints, spleen and blood. Our findings corroborate a previous study in which berberine was shown to alleviate collagen caused arthritis in rats by reducing the responses of Th17 cells (Yue *et al*., 2017).

To balance the excessive inflammatory milieu, IL-10 secreting T regulatory cells (Fontenot *et al*., 2003), which aid in immunological tolerance and immune suppression, are abundant. Optimum stability amid Treg and Th17 cells is necessary to preserve homeostasis since a ratio of Th17/Treg imbalance was demonstrated to be associated with the onset, severity of several inflammatory disorders, following the previous study's findings of septic arthritis (Noack & Miossec, 2014). Treatment with berberine (50, 100 and 200 mg/Kg) improved the balance between Treg and Th17cells, with a lower percentage of Th17 cells with CD4+ and RORγt and Treg cells with CD4, CD25, FOXp3 indicating feasible down-stream signaling pathways of Treg and Th17 cells with lower IL-21 and TGF-β levels in sepsis growth, perseverance. Berberine was shown to improve the equilibrium between Th17 and Treg cells, avoiding sepsis-induced arthritis caused by *S. aureus* for the first time.

The IL-17 produced by Th17 cells binding to its receptor activates the canonical NF-B and MAPK pathways, as well as JNK, and these transcription factors act in concert to increase the transcriptional activation of IL-17 target genes, resulting in chronic inflammation and autoimmune disease (Dey & Bishayi, 2017). Our study noticed greater levels of NF-κB and JNK expression in blood and tissues in diseased mice relative to the control group. The increased Th17 cells in a pathogenic environment, thus, may experience downstream signaling via NF-κB and MAPK, which would then exacerbate the current inflammatory process. Treatment with berberine (50, 100 and 200 mg/Kg) significantly reduced the amount of NF-κB and JNK expression in *S. aureus* ill mice with a lower number of Th17 cells, according to the findings. These findings may be due to lower stimulation of transcriptional IL-17 target genes, involved in the inflammatory responses associated with sepsis-induced arthritis in *S. aureus*-infected animals. The research reported here was consistent with previously gathered data that demonstrates that the impact of berberine on inflammatory cytokines in pig intestinal epithelial cells was that the NF-κB/MAPK signaling pathway's expression had decreased with berberine (Zhu *et al*., 2020).

The expression of IL-23R in pathogenic Th17 cells can be sustained through IL-23 upregulation, which aids in the continuation of inflammation (Monin & Gaffen, 2018). NF-κB signaling is necessary for the production of the IL-21 gene in T-cells, according to recent research. IL-21 is known to induce NF-κB activity in mice and has been demonstrated to do so in a STAT3-dependent manner. We discovered that therapy with berberine (50, 100 and 200 mg/Kg) concluded an insignificant fall in the serum IL-21 and the amount of Th17 cells with reduced NF-κB expression in the synovial joints, spleen and blood. Hence, elevated IL-21 levels at the initial stages of infection may be related to enhanced Th17 signaling via the NF-κB and/or JNK signaling pathways. The increased IL-21 signaling, in turn, upregulates NF-κB and JNK signaling, allowing the inflammatory signals to remain and aiding in the persistence of arthritis. Berberine (50, 100 and 200 mg/Kg) reduced these elevated levels of IL-21 by lowering Th17 signaling through NF-κB and JNK signaling pathways, reducing inflammation and *S. aureus*-induced arthritis. Berberine was found to improve intestinal barrier integrity and reduce inflammation, immunosuppression and oxidative stress in deoxynivalenol-challenged piglets in another investigation (Zhu *et al*., 2020).

This validates the pleiotropic involvement of transcriptional factors in T-cell response in sepsis-induced arthritis caused by *S. aureus*-infected mice, which was also observed in our investigation when the highest concentration of Tregs was discovered. The FoxP3 is necessary to sustain Treg immunosuppressive activity via the conventional NF-κB pathway. The results of our study depict, berberine (50, 100 and 200 mg/Kg) therapy reduced the Treg population with reduced JNK and NF-κB in the isolated Treg cells lysates of the blood and tissues, thereby minimizing the transcriptional functions of T-cell responses through lowered immunosuppression of FoxP3 in *S. aureus*-induced arthritis in mice. The treatment with berberine was also shown to help lower allergic inflammation in a mouse model for house dust mite allergic rhinitis by interfering with immune responses, as it lowered the number of CD4+, CD25+, Foxp3+ Treg cells (Kim *et al*., 2015).

Hyperactivation of NF-κB was shown to boost TGF-β/Smad activation, as it was found that NF-κB hyperactivation retained TGF-β/Smad stimulation in humans (Bettelli *et al*., 2006). TGF-β promotes osteoclast survival by activating the nuclear factor NF-κB, according to another study (Awasthi *et al*., 2009). The elevated TGF-β level might be a cause for arbitrating osteoclast survival leading to the progress of arthritis. Furthermore, higher JNK expression with increased Treg numbers and TGF-β concentration shows the importance of suppressing the functioning of Tregs. This links to the TGF-β-driven JNK signaling to maintain the regulation of the immune system (Chen *et al*., 2010).

The berberine (50, 100 and 200 mg/Kg) therapy lowered TGF-β levels, resulting in less hyperactivation of NF-κB activation. These findings will go a long way in helping to reduce swelling and the continuation of arthritis. Berberine was found to be efficient in reducing Treg function, decreasing TGF-β levels and reducing JNK expression in Treg isolated cells. Our findings corroborate previous research demonstrating that berberine protects against bleomycin-induced fibrosis by decreasing NF-κB-reliant on inflammatory and TGF-β1-mediated fibrotic processes (Chitra *et al*., 2013). The reduced expression of IL-1, IL-6, TGF-β, TNF- and imparted ICAM-1, as well as the reduced activities of phosphorylated JNK and PNF-B in postoperative abdominal adhesion and inflammation, was also observed in the berberine group, supporting our findings of reduced inflammatory responses

in sepsis-induced arthritis caused by *S. aureus* (Zhang *et al.*, 2014).

The interplay between RANK and RANKL is a powerful indication of osteoclastogenesis. We measured the expression of RANKL in Th17 cells from synovial joints in our research. It was discovered that SA groups had significantly higher RANKL expression than did the control group. Synovial Th17 cells with elevated RANKL expression are strongly related to mature osteoclast production and bone resorption (Kikuta *et al.*, 2013). Reduced expression of the RANKL in Th17 isolated cells from synovial joints was observed in the treatment groups, lowering the percentages of CD4+ in Th17 isolated cells and reducing osteoclast maturity and bone resorption. The results here are consistent with the other study, which discovered that berberine inhibited osteoclast differentiation via the inhibition of c-Fos and NFATc1 (Han & Kim, 2019).

Furthermore, IL-17, generated by Th17 cells when they are active, increases NF-κB/JNK signaling, which in turn boosts the expression of RANKL in the osteoblasts and synovial fibroblasts (Vernal & Garcia-Sanz, 2008). In the existence of macrophage-derived osteotropic factors such as IL-6, TNF-α and IL-1, the interaction of RANK-RANKL causes osteoclast distinction and stimulation, which leads to bone degradation. The generation of IL-17 from activated Th-17 cells was also reduced in our treatment groups, which suppressed NF-B or JNK signaling pathways, which in turn decreased RANKL expression in synovial fibroblasts and osteoclast cells. The treatment effects mitigated the damage caused by osteotropic factors coming from macrophages, such as IL-6, IL-1, or TNF-α, by decreasing osteoblast development and activation and so lowering bone degradation.

CONCLUSION

The berberine (50, 100 and 200 mg/Kg) treatments reduced Th17 cell proliferation and reduced CD4+ T cells induced by staphylococcal superantigenic stimulation, according to our findings. Reduced activity of Th17 cells inhibited the release of pro-inflammatory cytokines, including IL-17, hence suppressing the NF-κB/JNK signaling pathways. Further treatment with berberine leads to a decrease in the amount of Treg cells, implying the induction of anti-inflammatory effects *via* inhibition of NF-B or TGF-β driven JNK signaling pathways. According to our findings, reduced IL-21 secretion triggered down-regulation of NF-κB/JNK signaling and IL-23R in Th17 cells, regulating the duration of inflammatory responses. Furthermore, the treatment groups had lower RANKL expression due to NF-κB/JNK signaling downregulation in synovial fibroblasts and osteoblasts. Reduced RANKL expression resulted in decreased osteoclastogenesis, which reduced bone damage and improved arthritic symptoms. A possible strategy for combating disease severity with berberine treatment in *S. aureus* triggered septic arthritis in mice, which targets the Th17 driven NF-κB/JNK-RANKL axis.

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