Nitric oxide mediated *Staphylococcus aureus* pathogenesis and protective role of nanoconjugated vancomycin

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**ARTICLE INFO**

**Abstract**

**Objective:** To test the survival of *Staphylococcus aureus* (*S. aureus*) inside lymphocyte that contributes to the pathogenesis of infection and possible anti-inflammatory and antioxidative effect of nanoconjugated vancomycin against *in vivo* *S. aureus* infection in a dose and duration dependent manner. **Methods:** *5 × 10⁶* CFU/mL vancomycin–sensitive *S. aureus* (VSSA) and vancomycin–resistive *S. aureus* (VRSA) were challenged in Swiss male mice for 3 days, 5 days, 10 days and 15 days, respectively. Bacteremia, inflammatory markers and oxidative stress related parameters were observed to evaluate the duration for development of VSSA and VRSA infection. *100 mg/kg bw/day* and *500 mg/kg bw/day* nanoconjugated vancomycin were administrated to VSSA and VRSA infected group for successive 5 days, 10 days and 15 days. Bacteremia, inflammatory markers and oxidative stress related parameters were observed to assess the effective duration of nanoconjugated vancomycin against VSSA and VRSA infection.

**Results:** The result revealed that *in vivo* VSSA and VRSA infection developed after 5 days of challenge by elevating the NO generation in lymphocyte and serum inflammatory markers. Administration with nanoconjugated vancomycin to VSSA and VRSA infected group at a dose of *100 mg/kg bw/day* and *500 mg/kg bw/day* respectively, for successive 10 days eliminated bacteremia, decreased NO generation in lymphocyte, serum inflammatory markers and increased antioxidant enzyme status. **Conclusions:** These findings suggest, in vivo challenge of VSSA and VRSA for 5 days can produce the highest degree of damage in lymphocyte which can be ameliorated by treatment with nanoconjugated vancomycin for 10 successive days.

1. Introduction

*Staphylococcus aureus* (*S. aureus*) is a major human pathogen causing significant morbidity and mortality in both community– and hospital– acquired infections[1]. It causes a diverse array of infections ranging from relatively minor skin and wound infections to more serious and life–threatening diseases such as pneumonia, endocarditis, osteomyelitis, arthritis, and sepsis. Concern over the emergence of multidrug–resistant strains has renewed interest in understanding the virulence mechanisms of this pathogen at the molecular level and in elucidating host defense elements that either provide protection from or limit infection[2,3].

*S. aureus* is generally not considered as a significant intracellular pathogen. *Staphylococci* have typically been regarded as non–invasive extracellular pathogens that damage host cells after adhering to the extracellular matrix. However, there is a growing evidence that *S. aureus* has the ability to invade and persist within eukaryotic cells. The ability of *S. aureus* to survive in the eukaryotic intracellular environment could explain several aspects of chronic staphylococcal diseases and long–term colonization. Internalization may provide a means of protection against host defenses and certain classes of antibiotics. Many staphylococcal infections which tend to become chronic (e.g., osteomyelitis and mastitis) are associated with multiple recurrences and do not resolve even in the...
presence of an adequate humoral immune response[4]. S. aureus has been shown to be ingested by non-professional phagocytes, such as mouse fibroblasts, mouse renal cells and bovine mammary epithelial cells[5,6]. S. aureus also has the ability to invade mouse and human osteoblast cell lines, as well as normal mouse and human osteoblasts[7,8].

Polymorphonuclear neutrophils (PMN) have long been thought to provide significant host defense against S. aureus infection primarily because patients who are neutropenic or who have congenital or acquired defects in PMN function are more susceptible to infection with this pathogen[9]. The possibility that phagocytes, particularly PMN, could facilitate S. aureus infection has been raised by other investigators[10-12]. In vitro studies from the 1950s and 1960s demonstrated that pathogenic strains of S. aureus could survive for long periods of time inside both PMN and monocytes isolated from different animals and humans[11]. These studies led investigators at the time to speculate that both intracellular survival and extracellular multiplication play important roles in the pathogenesis of S. aureus infections[9]. In this regard, recent experiments assessing invasion and intracellular survival of S. aureus in endothelial cells, epithelial cells, and osteoblasts have suggested that intracellular survival could contribute to the persistence of the pathogen in S. aureus-induced endocarditis, bovine mastitis, and osteomyelitis[13]. In vitro studies suggested that the ability of S. aureus to exploit the inflammatory response of the host by surviving inside PMN is a virulence mechanism for this pathogen and that modulation of the inflammatory response is sufficient to significantly alter morbidity and mortality induced by S. aureus infection[14]. Till now no studies have assessed whether survival of S. aureus inside lymphocyte occurs in vivo and whether this can promote infection.

Chitosan (CS), the deacetylated form of chitin, is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine linked in a β linkage[15]. CS has been reported to possess immune stimulating properties such as increasing accumulation and activation of macrophages and polymorphonuclearus, suppressing tumor growth, augmenting antibody responses and inducing production of cytokines[16]. CMC is synthesized from CS by carboxylation of the hydroxyl and amine groups[17]. In our previous laboratory report, we synthesized CMC–EDBE–FA nanoparticle based on carboxy methyl chitosan tagged with folic acid by covalently linkage through 2, 2' (ethylenedioxoy) bis-(ethyamine), vancomycin was loaded onto it called “nanoconjugated vancomycin” and observe it’s in vitro bactericidal activity against S. aureus[18]. In our recent laboratory report, we observed that CMC–EDBE–FA is non-toxic[19]. The present study was aimed to test the survival of S. aureus inside lymphocyte that contributes to the pathogenesis of infection; and possible anti-inflammatory and antioxidative effect of nanoconjugated vancomycin against in vivo S. aureus infection in a dose and duration dependent manner.

2. Materials and methods

2.1. Chemicals and reagents

Sodium dodecyl sulfates, 2,7-Dichlorofluorescin diacetate, S′, DTNB, standard reduced glutathione, glutathione reductase (GR), NADPH Na4, oxidized glutathione (GSSG) were purchased from Sigma Chemical Co., USA. Sodium chloride, ethylene diamine tetra acetate, tryptic soy broth, sodium azide, were purchased from Himedia, India. Tris–HCl, Tris buffer, KH2PO4, K2HPO4, sodium hydroxide, O–phenylenediamine, alcohol and other chemicals were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. All other chemicals were from Merck Ltd., SRL Pvt., Ltd., Mumbai and were of the highest grade available.

2.2. Animals

Experiments were performed using Swiss male mice 6–8 weeks old, weighing 20–25 g. The animals were fed standard pellet diet and water were given ad libitum and housed in polypopylene cage (Tarsion) in the departmental animal house with 12 h light:dark cycle, and the temperature of (25± 2) °C. The animals were allowed to acclimatize for one week. The animals used did not show any sign of malignancy or other pathological processes. Animals were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India, and approved by the Ethical Committee of Vidyasagar University.

2.3. Bacterial strain

Coagulase positive vancomycin–sensitive S. aureus (VSSA) and vancomycin–resistive S. aureus (VRSA) strains were isolated from human post operative pus sample[20]. These bacterial strains were grown at 37 °C overnight in tryptic soy broth. The bacterial culture was centrifuged at 15 000 rpm for 15 min. The pellet was resuspended and washed with sterile phosphate buffer saline (PBS). Using a UV–spectrophotometer (Schimadzu, USA) at an absorbance of 620 nm, the viable bacterial count was adjusted to approximately 1.0×10⁹ colony-forming units (CFU)/mL, which corresponded to an optical density of 1.6. The bacterial suspension was adjusted by serial dilution in PBS to final concentration of approximately 5×10⁹ in 100 μL of bacterial suspension[21].

2.4. Preparation of CMC–EDBE–FA nanoparticle and loading of vancomycin

CMC–EDBE–FA nanoparticle was prepared and vancomycin was loaded onto it according to our previous laboratory report[18].

2.5. Experimental design

2.5.1. Duration dependent study to develop VSSA and VRSA infection in mice

Mice were randomized into control and experimental groups and divided into three groups of twenty four animals each. Mice in group A served as control; group B served as VSSA challenged (100 μL of bacterial suspension containing 5×10⁹ CFU/mL) and group C served as VRSA challenged (100 μL, of bacterial suspension containing 5×10⁹ CFU/mL). The treatment was done intraperitoneally (i.p.). The dose was selected as per reported by Wardenburgas et al[22]. The experiment was terminated at the end of 3 days, 5 days, 10 days and 15 days and all animals were sacrificed by an intraperitoneal injection of sodium pentobarbital (60–70 mg/
2.5.2. In vivo effective dose determination study of nanoconjugated vancomycin against VSSA and VRSA infection

To determine the effective dose, 100 mg/kg bw/day and 500 mg/kg bw/day nanoconjugated vancomycin were treated to VSSA and VRSA infected mice for successive 5 days. Mice were divided into 7 groups of six animals each. Group I: Control; Group II: VSSA infection; Group III: VSSA infection +100 mg/kg bw/day nanoconjugated vancomycin; Group IV: VSSA infection+500 mg/kg bw/day nanoconjugated vancomycin; Group V: VRSA infection; Group VI: VRSA infection+100 mg/kg bw/day nanoconjugated vancomycin; Group VII: VRSA infection+500 mg/kg bw/day nanoconjugated vancomycin.

2.5.3. In vivo effective duration determination study of nanoconjugated vancomycin against VSSA and VRSA infection

To determine the effective duration, VSSA and VRSA infected mice were treated with their effective dose of nanoconjugated vancomycin, respectively for 5 days, 10 days and 15 days.

After sacrifice blood sample (n=6/group) was used for quantification of viable bacteria, preparation of serum and separation of lymphocyte for biochemical estimation of different parameters.

2.6. Separation of serum and lymphocyte

Serum was obtained by centrifugation of blood samples taken without anticoagulant at 1 500 g for 15 min, and was kept at −86 °C for the further estimation. Heparinized blood samples were used for the separation of lymphocyte. Lymphocytes were isolated from blood using standard isolation techniques[24]. Blood samples were diluted with equal amount of PBS (pH 7.0) buffer and then layered very carefully on the density gradient (Histopaque 1077, Sigma Chemical Co.) in 1:2 ratio. Centrifuged at 500 g for 20 min and the white milky layer of mononuclear cells i.e., lymphocytes was carefully removed. The layer was washed twice with the same buffer and centrifuged at 3 000 g for 10 min to get the required pellet of lymphocytes[25]. The pellets of lymphocytes were lysed in a hypotonic lysis buffer for 45 min at 37 °C and kept at −86 °C until biochemical estimations[25]. Intact cells were used for reactive oxygen species (ROS) generation.

2.7. Quantification of viable bacteria in blood

A total of 100 μL of blood was inoculated in 1.0 mL sterile tryptic soy broth with sterile disposable micro-tips, grown at 37 °C for overnight, plated onto tryptic soy agar and mannitol salt agar plates in triplicate and incubated at 37 °C for 24 h. Colonies were counted by dilution plating method, and expressed as CFU per mL.

2.8. Biochemical estimation

The ROS generation in lymphocyte was measured according to Schreck and Baeuerle[26] using cell permeant probe 2,7-Dichlorofluorescin di-acetate (DCFH2DA). For each experiment, fluorometric measurements at a wavelength of 550 nm were performed in triplicate and data were expressed as mean arbitrary unit of 2,7-Dichlorofluorescin (DCF) fluorescence intensity.

Nitrite (NO) generation and release was measured according to Sanai et al[27] using Griess reagent (containing 1 part of 1% sulfanilamide in 5% phosphoric acid, and 1 part of 0.1% of N-(1 naphthyl ethylene diamine dihydrochloride). The levels of NO were expressed as μmol/ml protein.

C-reactive protein (CRP) level in serum was estimated using a sandwich ELISA Kit (Tulip, Mumbai, India). The assay was performed as per the detailed instructions of the manufacturer. The levels of CRP were expressed as μg/dL.

Myeloperoxidase (MPO) activity of serum was measured using OPD as substrate according to KarMahapatra et al[28]. MPO activity was expressed as μmol/mg protein.

TNFa and IL–10 level in serum were measured using a sandwich ELISA Kit (eBioScience; R&D systems). The assay was performed as per the detailed instructions of the manufacturer. The detection limit of these assays was <5,1, and <4 for TNFa and IL–10, respectively. The levels of TNFa and IL–10 were expressed as pg/mL.

Lipid peroxidation in cell lysate was estimated as TBA reactive substances (TBARS) by the method of KarMahapatra et al[28]. The levels of lipid peroxidation were expressed in terms of μmol/ml protein.

Protein oxidation in cell lysate was monitored by measuring protein carbonyl contents and by derivatization with 2, 4–dinitrophenyl hydrazine (DNPH)[28]. Protein carbonyls content was expressed in terms of μmol/mg protein.

Reduced glutathione (GSH) estimation was performed using Ellman’s reagent[28], and the levels were expressed as μg of GSH/mg protein. The GSSG level was measured after dervationization of GSH with 2-vinylpyidine[28], and levels were expressed as μg of GSH/mg protein. Super oxide dismutase (SOD) activity was determined from its ability to inhibit the auto–oxidation of pyrogallol[28] and expressed as unit/mg protein.

Catalase (CAT) activity was measured from the rate of absorbance change per min[28], and expressed in terms of μmol H2O2 consumed/min/mg protein. The glutathione peroxidase (GPx) activity, GR activity, and glutathione–s-transferase (GST) activity were all measured by the method of KarMahapatra et al[28]. The activity of GPx, GR and GST were expressed in terms of n mol NADPH consumed/min/mg protein. Protein was determined according to Lowry et al[29] using bovine serum albumin as standard.

2.9. Statistical analysis

The data were expressed as mean±standard error, n=6. Comparisons of the means of control, and experimental groups were made by two–way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with multiple comparison t-tests, P< 0.05 as a limit of significance.

3. Results

3.1. Development of VSSA and VRSA infection in mice
From our study it was observed that, viable bacterial count in blood was increased significantly after 3 days, 5 days, 10 days and 15 days of VSSA and VRSA challenge, respectively. Maximum increased viable bacteria count was found after 5 days of VSSA and VRSA challenge (Table 1). Maximum viable bacteria count was reduced significantly (P<0.05) by 118.97% in VSSA and 158.12% in VRSA infected group, respectively, after 100 mg/kg bw/day and 500 mg/kg bw/day nanoconjugated vancomycin treatment (Table 2). SOD, CAT, GPx, GR and GST activity were decreased significantly (P<0.05) by 39.71%, 60.34% in VSSA infected group, respectively; while by 26.79%, 40.02% in VRSA infected group, respectively, after 100 mg/kg bw/day and 500 mg/kg bw/day nanoconjugated vancomycin treatment (Table 2). GSH level was decreased significantly (P<0.05) by 97.35%, 66.95% in VSSA infected group, respectively; while by 119.10%, 95.89% in VRSA infected group, respectively (Table 2). GSSG level was increased significantly (P<0.05) by 49.95%, 108.91%, 102.71%, 98.81% and 69.23%, 143.67%, 135.73%, 128.65% after 3 days, 5 days, 10 days and 15 days of VSSA and VRSA challenge, respectively (Table 1). Serum CRP level and MPO activity were significantly increased (P<0.05) by 55.44%, 103.52%, 100.59%, 98.21%, and 56.54%, 82.72%, 80.28%, 79.03% after 3 days, 5 days, 10 days and 15 days of VSSA challenge, respectively; while by 81.69%, 150.65%, 140.92%, 148.84%, and 77.55%, 91.45%, 87.01%, 85.07% after 3 days, 5 days, 10 days and 15 days of VRSA challenge, respectively (Table 1). Serum IL-10 level was significantly decreased (P<0.05) by 51.16%, 39.71% in VSSA infected group, respectively; while by 42.61%, 36.49% and 35.47%, 28.9% and 55.44% in VRSA infected group respectively after 100 mg/kg bw/day and 500 mg/kg bw/day nanoconjugated vancomycin treatment (Table 2). SOD, CAT, GPx, GR and GST activity were increased significantly (P<0.05) by 134.69%, 88.25%, 77.56%, 48.25%, 69.38% and 114.61%, 64.62%, 63.56%, 44.35%, 52.54% in VSSA infected group, respectively; while by 106.52%, 80.61%, 61.62%, 55.17%, 52.62% and 138.66%, 120.26%, 81.65%, 87.07%, 88.47% in VRSA infected group, respectively, after 100 mg/kg bw/day and 500 mg/kg bw/day nanoconjugated vancomycin treatment (Table 2). Maximal effects of all these inflammatory parameters were observed at 100 mg/kg bw/day nanoconjugated vancomycin treatment on VSSA infected group and 500 mg/kg bw/day nanoconjugated vancomycin treatment on VRSA infected group.

3.2. In vivo effective dose determination of nanoconjugated vancomycin against VSSA and VRSA infection

It was observed that, viable bacterial count in blood of VSSA and VRSA infected group was decreased significantly after 100 mg/ kg bw day and 500 mg/ kg bw/ day nanoconjugated vancomycin treatment (Table 2). NO generation and release were increased significantly (P<0.05) by 43.92%, 95.76%, 94.31%, 94.72% and 42.29%, 82.06%, 75.44%, 77.93% after 3 days, 5 days, 10 days and 15 days of VSSA challenge, respectively; while by 53.34%, 107.15%, 102.28%, 101.97% and 49.94%, 101.55%, 92.63%, 96.75% after 3 days, 5 days, 10 days and 15 days of VRSA challenge, respectively (Table 1). ROS generation and release were increased significantly (P<0.05) by 43.83%, 39.53% in VSSA infected group, respectively; while by 40.33%, 46.93% in VRSA infected group, respectively, after 100 mg/kg bw/day and 500 mg/kg bw/day nanoconjugated vancomycin treatment (Table 2). Maximum increased viable bacteria count was found after 5 days, 10 days and 15 days of VSSA and VRSA challenge, respectively (Table 1). Serum CRP level and MPO activity were significantly increased (P<0.05) by 55.44%, 103.52%, 100.59%, 98.21%, and 56.54%, 82.72%, 80.28%, 79.03% after 3 days, 5 days, 10 days and 15 days of VSSA challenge, respectively; while by 81.69%, 150.65%, 140.92%, 148.84%, and 77.55%, 91.45%, 87.01%, 85.07% after 3 days, 5 days, 10 days and 15 days of VRSA challenge, respectively (Table 1). Serum IL-10 level was significantly decreased (P<0.05) by 51.16%, 39.71% in VSSA infected group, respectively; while by 42.61%, 36.49% and 35.47%, 28.9% and 55.44% in VRSA infected group respectively after 100 mg/kg bw/day and 500 mg/kg bw/day nanoconjugated vancomycin treatment (Table 2). GSH level was decreased significantly (P<0.05) by 97.35%, 66.95% in VSSA infected group, respectively; while by 119.10%, 95.89% in VRSA infected group, respectively (Table 2). GSSG level was increased significantly (P<0.05) by 49.95%, 108.91%, 102.71%, 98.81% and 69.23%, 143.67%, 135.73%, 128.65% after 3 days, 5 days, 10 days and 15 days of VSSA and VRSA challenge, respectively (Table 1). Maximum effects of all these inflammatory parameters were found after 5 days of VSSA and VRSA challenge.

3.3. In vivo effective duration determination of nanoconjugated vancomycin against VSSA and VRSA infection

Viable bacterial count in blood of VSSA and VRSA...
infected group was decreased significantly in duration dependent manner after treatment with 100 mg/kg bw/ day and 500 mg/kg bw/day nanoconjugated vancomycin and total elimination of bacteremia was observed after 10 days successive treatment (Table 3). NO generation and release were increased significantly (P<0.05) by 114.92%, 98.97%, 64.60% and 104.52%, 84.68%, 82.30% in VSSA infected group, respectively, after 5 days, 10 days and 15 days of infection; while in case of VSSA infected group these were increased significantly (P<0.05) by 135.11%, 119.38%, 85.04% and 120.76%, 101.14%, 98.69%. Treatment of 100 mg/kg bw/day nanoconjugated vancomycin in VSSA infected group decreased NO generation and release significantly (P<0.05) by 40.52%, 44.32%, 23.61% and 30.87%, 36.09%, 26.78%; and treatment of 500 mg/kg bw/ day nanoconjugated vancomycin in VSSA infected group decreased NO generation and release significantly (P<0.05) by 39.91%, 44.23%, 20.99% and 31.05%, 43.22%, 30.07% after 5 days, 10 days and 15 days successive treatment, respectively (Table 3). Serum CRP level and MPO activity were increased significantly (P<0.05) by 76.16%, 64.4%, 43.41% and 90.06%, 67.52%, 45.93% in VSSA infected group, respectively, after 5 days, 10 days and 15 days of infection; while in case of VSSA infected these were increased significantly (P<0.05) by 108.64%, 80.69%, 59.94% and 134.83%, 113.27%, 94.99%. Treatment of 100 mg/ kg bw/day nanoconjugated vancomycin in VSSA infected group decreased CRP level and MPO activity significantly (P<0.05) by 30.72%, 36.77%, 25.06% and 35.66%, 24.75%; and treatment of 500 mg/kg bw/day nanoconjugated vancomycin in VSSA infected group decreased CRP level and MPO activity significantly (P<0.05) by 50.06%, 64.66%, 45.18% and 60.51%, 64.79%, 60.51%.

### Table 1

**Inflammatory markers in lymphocyte and serum of VSSA and VRSA challenged group.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>VSSA infection</th>
<th>VSSA infection +NV</th>
<th>VRSA infection</th>
<th>VRSA infection +NV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum NO release (μM/mg protein)</td>
<td>21.08±0.37</td>
<td>21.11±0.32</td>
<td>21.04±0.28</td>
<td>21.00±0.27</td>
<td>30.00±0.32*</td>
</tr>
<tr>
<td>Serum MPO activity (μM/mg protein)</td>
<td>21.08±0.37</td>
<td>21.11±0.32</td>
<td>21.04±0.28</td>
<td>21.00±0.27</td>
<td>30.00±0.32*</td>
</tr>
</tbody>
</table>

### Table 2

**Inflammatory markers, lipid peroxidation, protein oxidation, glutathione cycle and anti-oxidant enzyme status in vancomycin treated VSSA and VSSA infected group for 5 days.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
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<th>VRSA infection</th>
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</tr>
</tbody>
</table>

Values are expressed as mean±SEM, *n=6*, indicates significant difference (P<0.05) compared with control group.

### Table 3

**Inflammatory markers, lipid peroxidation, protein oxidation, glutathione cycle and anti-oxidant enzyme status in VSSA and VSSA infected group for 5 days, 10 days and 15 days.**

<table>
<thead>
<tr>
<th>Parameter</th>
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<td>21.00±0.27</td>
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</tr>
</tbody>
</table>

Values are expressed as mean±SEM, *n=6*, indicates significant difference (P<0.05) compared with control group, # indicates significant difference (P<0.05) compared with infected control group.

### Table 4

**Inflammatory markers, lipid peroxidation, protein oxidation, glutathione cycle and anti-oxidant enzyme status in VSSA and VSSA infected group for 5 days, 10 days and 15 days.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
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<th>VSSA infection +NV</th>
<th>VRSA infection</th>
<th>VRSA infection +NV</th>
</tr>
</thead>
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<td>Serum NO release (μM/mg protein)</td>
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<td>21.11±0.32</td>
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Values are expressed as mean±SEM, *n=6*, indicates significant difference (P<0.05) compared with control group, # indicates significant difference (P<0.05) compared with infected control group.
28.67% and 31.87%, 32.3%, 29.04% after 5 days, 10 days and 15 days successive treatment, respectively (Table 3). Serum TNF-α level was increased significantly (P<0.05) by 119.53%, 93.09%, 91.57% in VSSA infected group after 5 days, 10 days and 15 days of infection; while in case of VRSA infected group these were increased significantly (P<0.05) by 125.42%, 98.95%, 97.58%. Treatment of 100 mg/kg bw/day nanomucjugated vancomycin in VSSA infected group decreased TNF-α level significantly (P<0.05) by 35.96%, 38.39%, 26.47%; and treatment of 500 mg/kg bw/day nanomucjugated vancomycin in VRSA infected group decreased TNF-α level significantly (P<0.05) by 22.78%, 37.26%, 25.66% after 5 days, 10 days and 15 days successive treatment, respectively (Table 3). Serum IL-10 level was decreased significantly (P<0.05) by 52.93%, 35.12%, 33.21% in VSSA infected group after 5 days, 10 days and 15 days of infection; while in case of VRSA infected group these were decreased significantly (P<0.05) by 66.25%, 48.53%, 50.00%. Treatment of 100 mg/kg bw/day nanomucjugated vancomycin in VSSA infected group increased IL-10 level significantly (P<0.05) by 34.65%, 40.95%, 32.50%; and treatment of 500 mg/kg bw/day nanomucjugated vancomycin in VRSA infected group increased IL-10 level significantly (P<0.05) by 55.23%, 64.64%, 61.12% after 5 days, 10 days and 15 days successive treatment, respectively (Table 3). Maximum protective effect was found after 10 days of nanomucjugated vancomycin treatment on both VSSA and VRSA infection.

MDA and PC level were increased significantly (P<0.05) by 157.48%, 144.73%, 143.04% and 153.05%, 115.73%, 115.03% in VSSA infected group, respectively, after 5 days, 10 days and 15 days of infection; while in case of VRSA infected group these were increased significantly (P<0.05) by 185.02%, 175.57%, 176.79% and 161.01%, 122.76%, 121.49%. Treatment of 100 mg/kg bw/day nanomucjugated vancomycin in VSSA infected group decreased MDA and PC level significantly (P<0.05) by 44.61%, 56.18%, 47.49% and 31.43%, 47.40%, 45.38% after 5 days, 10 days and 15 days successive treatment, respectively (Table 3). GSH level was decreased significantly (P<0.05) by 46.83%, 40.06%, 36.16% and 61.63%, 49.93%, 43.42% in VSSA and VRSA infected group, respectively, after 5 days, 10 days and 15 days of infection. Treatment of 100 mg/kg bw/day nanomucjugated vancomycin in VSSA infected group increased GSH level significantly (P<0.05) by 22.78%, 37.26%, 25.66% after 5 days, 10 days and 15 days of infection respectively (Table 3). SOD, CAT, GPx, GR, GST activity were decreased significantly (P<0.05) by 55.38%, 43.25%, 39.86%; 59.38%, 55.67%, 53.56%; 58.01%, 47.98%, 44.71%; 44.77%, 44.76%, 44.07%; 51.77%, 46.2%, 39.56% in VSSA infected group, respectively, after 5 days, 10 days and 15 days of infection; while in case of VRSA infected group these were decreased significantly (P<0.05) by 63.43%, 49.97%, 46.29%; 72.64%, 65.93%, 64.98%; 60.72%, 57.63%, 52.38%; 64.14%, 53.77%, 49.5%; 57.02%, 51.43%, 44.78%. Treatment of 100 mg/kg bw/day nanomucjugated vancomycin in VSSA infected group increased SOD, CAT, GPx, GR, GST activity significantly (P<0.05) by 64.97%, 70.10%, 46.68%; 54.69%, 91.50%, 55.3%; 61.36%, 80.74%, 61.70%; 44.93%, 56.08%, 40.56%; 60.39%, 65.17%, 24.58%; and treatment of 500 mg/kg bw/day nanomucjugated vancomycin in VRSA infected group increased SOD, CAT, GPx, GR, GST activity significantly (P<0.05) by 76.08%, 79.76%, 51.61%; 86.4%, 109.23%, 66.37%; 79.38%, 119.54%, 63.88%; 56.67%, 67.97%, 37.75%; 55.55%, 72.19%, 26.91% after 5 days, 10 days and 15 days successive treatment, respectively (Table 3). Maximum protective effect was found after 10 days of nanomucjugated vancomycin treatment on both VSSA and VRSA infection.

4. Discussion

S. aureus resistant to antibiotics appears within a few years after the onset of the antibiotic epoch and currently spans all known classes of antibiotics[30]. Increasing resistance of S. aureus to vancomycin in developed and developing countries is alarming. Vancomycin resistance in S. aureus highlights the need for development of new and novel anti–VRSA antibiotics[31]. To develop the anti–VRSA antibiotics we used carboxymethyl chitosan conjugated folic acid nanoparticles, to which vancomycin was loaded and charged against vancomycin sensitive and resistant S. aureus infected mice.

To the best of our knowledge, this is the first report on survival of S. aureus inside lymphocyte and launched of an anti-inflammatory and antioxidative antibiotics against in vivo S. aureus infection. Serum CRP and MOP are the most important inflammatory markers of inflammation[32]. Inflammation is a multiple process mediated by activating inflammatory or immune cells. During the inflammation process, lymphocytes play a central role in managing many different immunopathological phenomena including the overproduction of proinflammatory cytokines and inflammatory mediators such as TNF–α, iNOS, NO[33]. Under inflammatory conditions, immune cells are also stimulated by adhesion molecule activation signals in order to enhance the migration capacity to inflamed tissue and finally to form heterotypic cell clustering among the immune cells, endothelial cells and inflamed cells[34]. To pro–inflammatory cytokines and mediators, TNF–α and NO play important roles in inflammatory process, so do anti–inflammatory factors such as IL–10. Then the change on them might be used for explaining the anti–inflammatory effects of many drugs. NO is from arginine after the activation of iNOS and it is an important effect molecule involved in immune regulation and defense[35].

It was observed from our study that blood bacteremia count, nitrate generation in lymphocyte, serum NO release, CRP level, MOP activity, TNF–α level were increased; and serum IL–10 level was decreased significantly in VSSA and VRSA challenged experimental group after 5 days, 5 days, 10 days and 15 days; but maximal effects were obtained after 5 days. NO, MOP, CRPs, TNF–α and IL–10 are inflammatory markers. So, maximum elevation of this marker after 5 days
of VSSA and VRSA challenge suggests the development of infection.

On the other hand, the result of our study reveals that blood bacteremia count, nitrate generation in lymphocyte, serum NO release, CRP level, MOP activity, TNF-α level were decreased; and serum IL-10 level was increased significantly in 100 mg/kg bw/day and 500 mg/kg bw/day nanoconjugated vancomycin treated VSSA and VRSA infected group but maximal effects were obtained in 100 mg/kg bw/day nanoconjugated vancomycin treated VSSA and VRSA infected group and 500 mg/kg bw/day nanoconjugated vancomycin treated VSSA infected group. So, it indicates that 100 mg/kg bw/day and 500 mg/kg bw/day nanoconjugated vancomycin have anti-inflammatory effect on VSSA and VRSA infection, respectively.

Our study reveals that, on 10 days there were no viable bacteria in blood indicating the total elimination of bacteria from blood. It was observed that nitrate generation in lymphocyte, serum nitric oxide release, CRP level, MOP activity, TNF-α level was decreased and serum IL-10 level was increased significantly in 100 mg/kg bw/day and 500 mg/kg bw/day nanoconjugated vancomycin treated VSSA and VRSA infected group, respectively, in a duration dependent fashion, but maximal effects were obtained after 10 days. So, it clearly indicates that 100 mg/kg bw/day and 500 mg/kg bw/day nanoconjugated vancomycin treatment for successive 10 days eliminates the bacteremia in VSSA and VRSA infected mice, respectively.

The results of dose and duration dependent study reveal that, MDA and PC levels were increased significantly due to VSSA and VRSA infection that were ameliorated after treatment of nanoconjugated vancomycin. It may be due to the generation of free radicals which may react with protein in addition to lipids. Protein carbonyls formation has been indicated to be an earlier marker of protein oxidation. Oxidation of protein may be due to either excessive oxidation of proteins or decreased capacity to clean up oxidative damaged proteins. Oxidative modification of proteins may lead to the structural alteration and functional inactivation of many enzyme proteins[36], as evidenced by the decreased activity of different antioxidant enzymes like SOD, CAT, GPx, GR, and GST.

Glutathione, an important cellular reductant, is involved in protection against free radicals, peroxides, and toxic compounds in cellular systems[37]. In the present study, the GSH levels were decreased and GSSG levels were increased due to VSSA and VRSA infection that were ameliorated after treatment of nanoconjugated vancomycin. The decreased GSH levels represent its increased utilization that may be due to increasing level of lipid oxidation products which may be associated with less availability of NADPH required for the activity of GR to transform GSSG to GSH[38]. In our present study, the increasing levels of GSSG and decreasing GR activity may support the explanation.

In our study, glutathione dependent enzymes and antioxidant enzyme status were decreased significantly in VSSA and VRSA infection which were ameliorated after nanoconjugated vancomycin treatment. GPx and CAT act as preventive antioxidant enzymes and SOD, a chain breaking antioxidant enzyme, play an important role in protection against the deleterious effect of lipid peroxidation[39]. The activity of SOD was decreased significantly in VSSA and VRSA infection. It may be due to the generation of reactive nitrogen species which dismutated to H2O2 by SOD[40]. Where the SOD stops its action, there the CAT exerts its function. The primary role of CAT is to scavenge H2O2 that has been generated by free radicals or by SOD in removal of superoxide anions and to convert it to water[41]. In our present study, the decreasing in CAT activity may be related to excess H2O2 production from SOD inhibition[42]. The maximum decreasing activity of SOD and CAT in lymphocyte due to VSSA and VRSA infection may be due to maximum utilization to scavenge the free radical generation. GPx works non-specifically to scavenge and decompose excess hydroperoxides including H2O2, which may be prevalent under oxidative stress[43]. GST mainly detoxifies electrophilic compounds and has a well-established role in protecting cells as a free radical scavenger along with glutathione[44]. In the present study, the decreasing level of GSH and decreased activity of GSH—dependent enzymes, i.e. GPx, GR, and GST in lymphocyte due to VSSA and VRSA infection may be due to increased utilization to scavenge the free radical generation.

In conclusion, the lymphocytes are susceptible to S. aureus infection through the increased production of nitric oxide, TNF-α which leads to decreased antioxidant status in cell. In vivo challenge of VSSA and VRSA for 5 days can produce the highest degree of damage in this important immunological cell, lymphocyte. In vivo treatment of nanoconjugated vancomycin treatment can ameliorates this type of staphylococcal damage and eliminates bacteremia.

Acknowledgements

The authors express gratefulness to the Department of Biotechnology, Government of India for funding; Indian Institute of Technology, Kharagpur and Vidyasagar University, Midnapore for providing the facilities to execute these studies.

Conflict of interest statement

We declare that we have no conflict of interest.

References


