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

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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:** 5×10^6 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 and inflammatory 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 5 days. Bacteremia, inflammatory parameters and oxidative stress related parameters were tested to observe the effective dose of nanoconjugated vancomycin against VSSA and VRSA infection. Nanoconjugated vancomycin was treated at a dose of 100 mg/kg bw/day and 500 mg/kg bw/day, respectively, to VSSA and VRSA infected group for successive 5 days, 10 days and 15 days. Bacteremia, inflammatory parameters 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 bacterimia, 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

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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[10]. 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 vivo* 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 polymorphonucleus, 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′ (ethylenedioxy) bis-(ethylamine), 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 di-acetate, 5′, DTNB, standard reduced glutathione, glutathione reductase (GR), NADPH Na_4, 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, KH_2PO_4, K_2HPO_4, 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 polypropylene cage (Tarson) in the departmental animal house with 12 h light:dark cycle, and the temperature of (25±2) ℃. 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 ℃ 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 1.0×10^9 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 5×10^6 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 5×10^6 CFU/mL) and group C served as VRSA challenged (100 μL of bacterial suspension containing 5×10^6 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/

kg bw)[23].

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 ℃ 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 ℃ and kept at −86 ℃ 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 ℃ for overnight, plated onto tryptic soy agar and mannitol salt agar plates in triplicate and incubated at 37 ℃ for 24 h. Colonies were counted by dilution platting 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-C-1 napthyl ethylene diamine dihydrochloride). The levels of NO were expressed as μmol/mg 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.

TNF-α 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 TNF-α and IL-10, respectively. The levels of TNF-α 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/mg 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 derevatization 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 pyrogalol[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 H_2O_2 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). ROS generation in lymphocyte was increased by 3.71%, 4.40%, 4.28%, 4.16% and 3.98%, 4.8%, 4.81%, 4.76% after 3 days, 5 days, 10 days and 15 days of VSSA and VRSA challenge, respectively; but all these increment were insignificant (Table 1).

NO generation and release were significantly increased (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). Serum CRP level and MOP 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 TNF- α level was significantly increased (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 IL-10 level was significantly decreased (P<0.05) by 32.83%, 56.68%, 44.53%, 44.93% and 39.47%, 63.63%, 48.29%, 49.51% 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.

3.2. 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 97.35%, 66.95% in VSSA infected group, respectively; while by 119.10%, 95.89% in VRSA infected group, respectively (Table 2). NO generation and release were significantly decreased (P<0.05) by 35.47%, 28.9% and 28.81%, 33.08% in VSSA infected group, respectively whereas 31.99%, 41.45% and 34.20%, 43.24% in VRSA infected group, respectively, after 100 mg/kg bw/day and 500 mg/kg bw/ day nanoconjugated vancomycin treatment (Table 2). Serum CRP level and MOP activity were increased significantly (P<0.05) by 98.27%, 52.42% in VSSA infected group, respectively; while by 124.62%, 129.54% in VRSA infected group, respectively (Table 2). CRP level and MPO activity were significantly decreased (P<0.05) by 42.61%, 36.49% and 31.29%, 22.41% in VSSA infected group, respectively; while by 28.18%, 42.40% and 29.39%, 43.52% in VRSA infected group, respectively, after 100 mg/kg bw/day and 500 mg/kg bw/day nanoconjugated vancomycin treatment (Table 2). Serum TNF- α level was increased significantly (P<0.05) by 118.97% in VSSA infected group and 158.12% in VRSA infected group, respectively (Table 2). TNF- α level was significantly decreased (P<0.05) by 40.13%, 36.60% in VSSA infected group, respectively; while by 29.53%, 49.54% in VRSA infected group, respectively, after 100 mg/kg bw/ day and 500 mg/kg bw/day nanoconjugated vancomycin treatment (Table 2). Serum IL-10 level was decreased significantly (P<0.05) by 40.87% in VSSA infected group and 56.76% in VRSA infected group, respectively (Table 2). IL-10 level was significantly increased (P<0.05) by 51.16%, 33.84% in VSSA infected group, respectively; while by 80.16%, 97.42% 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.

What's more, malondialdehyde (MDA) and protein carbonyls contents (PC) level were increased significantly (P<0.05) by 111.45%, 125.37% in VSSA infected group, respectively; while by 157.18%, 164.75% in VRSA infected group, respectively (Table 2). MDA level was decreased 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). PC level was decreased significantly (P<0.05) by 39.71%, 35.34% in VSSA infected group, respectively; while by 39.12%, 52.47% 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 49.73% in VSSA infected group and 54.14% in VRSA infected group, respectively (Table 2). GSH level was increased significantly (P<0.05) by 73.95%, 68.07% in VSSA infected group, respectively; while by 50.71%, 90.46% in VRSA infected group, respectively, after 100 mg/kg bw/day and 500 mg/kg bw/day nanoconjugated vancomycin treatment (Table 2). GSSG level was increased significantly (P<0.05) by 99.49% in VSSA infected group and 110.42% in VRSA infected group, respectively (Table 2). GSSG level was decreased significantly (P<0.05) by 35.09%, 28.03% 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). SOD, CAT, GPx, GR and GST activity were decreased significantly (P<0.05) by 63.42%, 56.27%, 46.98%, 41.13% and 50.19% in VSSA infected group, respectively where as 67.32%, 65.37%, 53.66%, 57.18% and 52.13% in VRSA infected group, respectively (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 oxidative 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.

3.3. 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 VRSA 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 VRSA 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 MOP 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 VRSA infected group 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 29.96%, 35.66%, 24.75%; and treatment of 500 mg/kg bw/day nanoconjugated vancomycin in VRSA infected group decreased CRP level and MPO activity significantly (P<0.05) by 33.05%, 36.86%,

Table 1

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

Parameter		Control				VSSA infection				VRSA infection			
		3 days	5 days	10 days	15 days	3 days	5 days	10 days	15 days	3 days	5 days	10 days	15 days
Viable bacteria count in blood (CFU/mL)		0	0	0	0	119 327.00±2 208.11*	215 183.00±1 613.21*	212 417.00±2 399.03*	213 789.00±2 394.03*	151 615.0±2 038.03*	289 189.00±1 958.18*	286 811.00±1 945.73*	287 812.00±2 044.73*
Serum	NO release (μM/mg protein)	21.08±0.37	21.11±0.32	21.04±0.28	21.00±0.27	30.00±0.32*	38.42±0.28*	36.91±0.38*	37.37±0.39*	31.61±0.29*	42.54±0.36*	40.53±0.28*	41.32±0.32*
	CRP (μg/dL)	5.41±0.17	5.31±0.20	5.40±0.18	5.63±0.20	8.41±0.21*	10.81±0.22*	10.83±0.28*	11.16±0.29*	9.83±0.40*	13.32±0.30*	13.01±0.31*	14.01±0.31*
	MPO (μM/mg protein)	1.53±0.02	1.63±0.02	1.55±0.02	1.51±0.02	2.39±0.03*	2.97±0.03*	2.79±0.03*	2.70±0.03*	2.71±0.03*	3.11±0.03*	2.89±0.03*	2.79±0.03*
	TNF-α (pg/mL)	80.13±2.96	87.72±3.27	85.01±3.16	83.10±3.01	120.15±3.95*	183.26±5.63*	172.33±5.64*	165.12±5.31*	135.63±4.89*	213.75±7.17*	200.40±5.46*	190.01±5.13*
	IL-10 (pg/mL)	210.32±5.36	220.01±6.21	215.12±6.07	218.12±6.03	141.27±4.96*	95.31±3.01*	119.32±4.68*	120.12±4.65*	127.31±3.89*	80.017±2.79*	111.23±3.71*	110.12±3.70*
Lymphocyte	ROS generation (a.u of DCF fluorescence intensity)	1.48±0.04	1.50±0.04	1.50±0.04	1.49±0.04	1.54±0.04	1.57±0.04	1.56±0.04	1.55±0.04	1.54±0.05	1.373±0.047	1.57±0.05	1.56±.04
	NO generation (μM/mg protein)	17.03±0.33	17.29±0.28	17.12±0.35	17.17±0.36	24.51±0.30*	33.85±0.36*	33.27±0.29*	33.43±0.29*	26.12±0.29*	35.82±0.35*	34.64±0.28*	34.67±0.28*

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

Table 2

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

Parameter		Control	VSSA infection control	VSSA infection +100 mg/kg bw NV	VSSA infretion +500 mg/kg bw NV	VRSA infection control	VRSA infection +100 mg/kg bw NV	VRSA infection +500 mg/kg bw NV
Viable bacteria count in blood (CFU/mL)		0	209 735.00±1 807.13*	66 367.00±2 337.12#	69 367.00±1 697.37#	292 189.00±2 258.18*	143 116.00±1645.74#	71 810.00±1 938.04#
Serum	NO release (μM/mg protein)	23.37±0.38	39.02±0.39*	26.11±0.38#	28.01±0.40#	45.78±0.40*	30.12±0.38#	25.99±0.40#
	CRP (μg/dL)	5.04±0.20	9.99±0.20*	5.37±0.19#	6.34±0.18#	11.31±0.21*	8.13±0.17#	6.52±0.17#
	MPO (μM/mg protein)	0.93±0.03	1.42±0.40*	0.98±0.04#	1.10±0.03#	2.14±0.04*	1.51±0.03#	1.21±0.03#
	TNF-α (pg/mL)	90.57±1.93	198.32± 2.11*	118.74±1.87#	125.74±1.79#	233.78±4.71*	164.74±3.67#	117.96±2.79#
	IL-10 (pg/mL)	225.12±4.76	133.12±2.91*	201.23±3.79#	178.17±3.17#	97.34±2.61*	175.37±3.59#	192.17±3.63#
Lymphocyte	NO generation (μM/mg protein)	15.69±0.21	30.96±0.28*	19.98±0.24#	22.01±0.28#	34.37±0.28*	23.38±0.27#	20.12±0.28#
	MDA (μmol/mg protein)	57.27±1.29	121.10±3.28*	68.02±1.29#	73.23±1.31#	147.29±3.11*	87.89±2.09#	77.89±2.07#
	PC (μmol/mg protein)	20.12±0.38	45.35±0.50*	27.34±0.37#	29.32±0.38#	53.27±0.42*	32.43±0.35#	25.32±0.35#
	GSH (μg/mg protein)	13.00±0.32	6.54±0.22*	11.37±0.30#	10.99±0.30#	5.96±0.27*	8.99±0.24#	11.36±0.30#
	GSSG (μg/mg protein)	35.78±1.34	71.38±2.02*	46.33±1.81#	51.37±1.88#	75.29±2.03*	55.12±1.90#	45.16±1.78#
	SOD (Unit/mg protein)	66.64±2.23	24.38±1.02*	57.21±1.87#	53.32±1.88#	21.78±1.12*	44.98±1.78#	51.98±1.78#
	CAT (μmol H_2O_2 consumed/min/mg protein)	30.57±0.18	13.37±0.26*	25.17±0.15#	22.01±0.19#	10.59±0.26*	19.12±0.20#	23.32±0.19#
	GPx (n mol NADPH consumed/min/mg protein)	6.13±0.07	3.25±0.06*	5.77±0.06#	5.31±0.06#	2.84±0.04*	4.59±0.06#	5.16±0.05#
	GR (n mol NADPH consumed/min/mg protein)	21.02±0.41	12.37±0.31*	18.34±0.38#	17.86±0.30#	9.09±0.42*	14.10±0.21#	16.91±0.35#
	GST (n mol NADPH consumed/min/mg protein)	34.20±0.30	17.04±0.46*	28.86±0.53#	25.99±0.35#	16.37±0.24*	24.99±0.35#	30.86±0.53#

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 3

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

Parameter		Control			VSSAinfection			VSSA infection +NV			VRSA infection			VRSA infection +NV		
		5 days	10 days	15 days	5 days	10 days	15 days	5 days	10 days	15 days	5 days	10 days	15 days	5 days	10 days	15 days
Viable bacteria in blood count (CFU/mL)		0	0	0	225 728.00±2 305.26*	217 391.00±2 297.27*	210 381.00±1 985.12*	48 512.00±2 366.21#	0	0	298 118.00±2 132.98*	291 327.00±1 845.12*	287 937.00±1687.28*	59 307.00±2 156.10#	0	0
Serum	NO release (μM/mg protein)	18.48±0.34	18.22±0.39	18.30±0.37	37.79±0.41*	33.65±0.44*	33.36±0.43*	26.12±0.41#	21.51±0.35#	24.42±0.38#	40.79±0.41*	36.65±0.44*	36.36±0.43*	28.12±0.41#	20.81±0.35#	25.42±0.38#
	CRP (μg/dL)	6.28±0.19	6.14±0.12	6.05±0.19	10.99±0.21*	10.09±0.18*	8.68±0.20*	7.61±0.17#	6.38±0.17#	6.50±0.14#	13.01±0.21*	11.09±0.18*	9.68±0.20*	8.71±0.17#	7.00±0.16#	6.90±0.14#
	MPO (μM/mg protein)	1.12±0.03	1.093±0.02	1.02±0.02	2.12±0.04*	1.83±0.04*	1.49±0.03*	1.49±0.03#	1.18±0.03#	1.12±0.02#	2.62±0.04*	2.33±0.04*	1.99±0.03*	1.48±0.03#	1.58±0.03#	1.41±0.03#
	TNF-α (pg/mL)	101.87±2.39	102.37±2.41	99.87±3.19	223.64±2.97*	197.67±2.37*	191.32±3.88*	143.23±3.01#	121.78±2.23#	140.68±2.81#	229.64±3.06*	203.67±2.97*	197.32±3.98*	177.32±3.98#	127.78±2.47#	146.68±3.01#
	IL-10 (pg/mL)	300.27±5.99	298.27±5.96	297.78±5.77	141.35±3.69*	193.52±2.73*	198.88±3.87*	190.33±4.19#	272.76±4.49#	263.52±3.58#	101.35±3.49*	153.52±2.37*	148.88±3.27*	257.33±3.68#	252.76±4.29#	239.88±4.11#
Lymphocyte	NO generation (μM/mg protein)	14.87±0.28	14.71±0.24	14.67±0.26	31.96±0.27*	29.26±0.22*	24.16±0.26*	19.01±0.24#	16.29±0.23#	18.46±0.26#	34.97±0.26*	32.26±0.22*	27.16±0.26*	21.01±0.24#	17.99±0.23#	21.46±0.19#
	MDA (μmol/mg protein)	60.24±1.21	72.21±1.48	68.46±1.39	155.11±2.00*	176.73±2.01*	166.39±2.03*	102.23±1.17#	88.32±1.06#	94.62±.1.58#	171.70±2.03*	199.00±2.02*	189.50±2.02*	95.10±1.42#	87.20±1.32#	99.50±1.52#
	PC (μmol/mg protein)	26.39±1.08	28.29±1.15	28.94±1.14	66.78±1.82*	61.03±1.54*	62.23±1.42*	45.36±1.36#	32.01±1.01#	36.23±1.12#	68.88±1.84*	63.02±1.56*	64.10±1.75*	47.23±1.39#	33.15±1.11#	35.01±1.02#
	GSH (μg/mg protein)	12.62±0.21	10.23±0.22	10.23±0.23	6.48±0.22*	6.13±0.22*	6.53±0.22*	9.00±0.28#	9.63±0.28#	9.12±0.27#	4.84±0.21*	5.12±0.22*	5.79±0.23*	8.20±0.27#	8.96±0.27#	8.50±0.24#
	GSSG (μg/mg protein)	36.71±0.55	35.13±0.75	38.08±0.64	74.56±0.86*	70.87±0.95*	73.21±0.97*	47.89±0.85#	38.89±0.64#	51.02±0.75#	78.65±0.88*	74.78±0.94*	77.12±0.96*	48.98±0.89#	40.89±0.64#	50.21±0.75#
	SOD (Unit/mg protein)	77.86±0.92	75.73±0.75	76.30±0.82	34.74±0.58*	42.98±0.67*	45.89±0.58*	57.31±0.80#	73.11±0.89#	67.31±0.80#	28.47±0.56*	37.89±0.67*	40.98±0.59*	50.13±0.79#	68.11±0.89#	62.13±0.79#
	CAT (μmol H_2O_2 consumed/min/mg protein)	30.14±0.23	29.32±0.21	29.57±0.23	12.24±0.24*	13.00±0.18*	13.74±0.18*	18.94±0.23#	24.89±0.21#	21.33±0.24#	8.25±0.22*	9.99±0.20*	10.36±0.19*	15.37±0.19#	20.90±0.19#	17.23±0.14#
	GPx (nmol NADPH consumed/min/mg protein)	7.41±0.07	7.25±0.04	7.04±0.09	3.11±0.06*	3.77±0.07*	3.89±0.07*	5.02±0.09#	6.81±0.08#	6.29±0.09#	2.91±0.06*	3.07±0.06*	3.35±0.08*	5.22±0.10#	6.74±0.08#	5.49±0.07#
	GR (nmol NADPH consumed/min/mg protein)	21.34±0.17	22.21±0.18	22.09±0.18	9.651±0.174*	12.27±0.177*	12.36±0.15*	13.99±0.19#	19.15±0.16#	17.37±0.18#	7.65±0.16*	10.27±0.18*	11.16±0.15*	11.99±0.19#	17.25±0.17#	15.37±0.18#
	GST (nmol NADPH consumed/min/mg protein)	38.09±0.25	38.22±0.26	38.32±0.31	225 728.00±2 305.26*	20.56±0.28*	23.16±0.28*	29.47±0.24#	33.96±0.28#	28.85±0.27#	16.37±0.23*	18.56±0.21*	21.16±0.21*	25.47±0.30#	31.96±0.25#	26.85±0.27#

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 nanoconjugated 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 nanoconjugated 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 nanoconjugated 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 nanoconjugated 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 nanoconjugated 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 nanoconjugated vancomycin in VSSA infected group decreased MDA and PC level significantly (P<0.05) by 34.09%, 50.03%, 43.13% and 32.08%, 47.55%, 41.78%; and treatment of 500 mg/kg bw/day nanoconjugated vancomycin in VRSA 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 48.63%, 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 nanoconjugated vancomycin in VSSA infected group increased GSH level significantly (P<0.05) by 38.90%, 57.08%, 39.67%. Treatment of 500 mg/kg bw/day nanoconjugated vancomycin in VRSA infected group increased GSH level significantly (P<0.05) by 69.42%, 74.96%, 46.83% (Table 3). GSSG level was increased significantly (P<0.05) by 103.11%, 101.74%, 92.25% and 114.25%, 112.87%, 102.52% in VSSA and VRSA infected group, respectively, after 5 days, 10 days and 15 days of infection. Treatment of 100 mg/kg bw/day nanoconjugated vancomycin in VSSA infected group decreased GSSG level significantly (P<0.05) by 35.77%, 45.12%, 30.31%. Treatment of 500 mg/kg bw/day nanoconjugated vancomycin in VRSA infected group increased GSH level significantly (P<0.05) by 37.72%, 45.32%, 34.89% (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%; 54.77%, 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 nanoconjugated 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 nanoconjugated 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 nanoconjugated 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 3 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 bacterimia 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 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 bacterimia 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 dismuted to H_2O_2 by SOD[40]. Where the SOD stops its action, there the CAT exerts its function. The primary role of CAT is to scavenge H_2O_2 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 H_2O_2 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 H_2O_2, 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 bacterimia.

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Conflict of interest statement

We declare that we have no conflict of interest.

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