

Research Article

Staphylococcus aureus Infection Induced Oxidative Imbalance in Neutrophils: Possible Protective Role of Nanoconjugated Vancomycin

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Staphylococcus aureus infection causes oxidative stress in neutrophils. The immune cells use reactive oxygen species (ROS) for carrying out their normal functions while an excess amount of ROS can attack cellular components that lead to cell damage. The present study was aimed to test the protective role of nanoconjugated vancomycin against vancomycin-sensitive *Staphylococcus aureus* (VSSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) infection induced oxidative stress in neutrophils. VSSA- and VRSA-infection were developed in Swiss mice by intraperitoneal injection of 5×10^6 CFU/mL bacterial solutions. Nanoconjugated vancomycin was treated to VSSA- and VRSA-infected mice at its effective dose for 10 days. Vancomycin was treated to VSSA and VRSA infected mice at similar dose, respectively, for 10 days. The result reveals that in vivo VSSA and VRSA infection significantly increases the level of lipid peroxidation, protein oxidation, oxidized glutathione level, and nitrite generation and decreases the level of reduced glutathione, antioxidant enzyme status, and glutathione-dependent enzymes as compared to control group; which were increased or decreased significantly near to normal in nanoconjugated vancomycin-treated group. These findings suggest the potential use and beneficial protective role of nanoconjugated vancomycin against VSSA and VRSA infection induced oxidative imbalance in neutrophils.

1. Introduction

Staphylococcus aureus is a major human pathogen causing large variety of infections worldwide with prevalence rate ranging from 4.6–54.4% [1–5]. *S. aureus* causes superficial skin infections to life-threatening diseases such as endocarditis, sepsis and soft tissue, urinary tract, respiratory tract, intestinal tract, and bloodstream infections [6, 7]. 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 mean of protection against host defenses and certain classes of antibiotics. Staphylococcal infections are typically associated with death of tissue, and evidence suggests intracellular bacteria are capable of inducing apoptosis.

S. aureus-mediated apoptosis has been reported in epithelial cells [8–10], keratinocytes [11], endothelial cells [12, 13], and osteoblasts [14]. Wesson et al. demonstrated host caspases-8 and -3 to play a role in *S. aureus*-induced apoptosis, and caspase-8 is known to be associated with apoptosis triggered by engagement of death receptors [10]. Resistance of *S. aureus* to antibiotics appeared within a few years after the onset of the antibiotic era [15], and this problem has reached epic proportions owing to overuse and improper use of anti-biotics [16]. *S. aureus* resistance to antibiotics currently spans all known classes of natural and synthetic compounds [17]. Increasing resistance of *S. aureus* to last line of drug, that is, vancomycin highlights the need for either the development of new and novel antibiotics [18, 19] or the improvement of efficacy of established antibiotics by the

development of new agents capable of enhancing antibiotic activity [20].

Chitin, a natural biopolymer, is the major structural component of invertebrates like crab, shrimp, shells, and the cell walls of fungi. Chitosan (CS), the deacetylated form of chitin, is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine linked in a β linkage [21–24]. Carboxymethyl chitosan (CMC) is a linear polysaccharide composed of β (1,4) glycosidic linkages between 6-carboxymethyl-D-glucosamine monomers. CMC is synthesized from CS by carboxylation of the hydroxyl and amine groups [25]. 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, complex is called “nanoconjugated vancomycin” and observe it's bactericidal activity against *S. aureus* [26]. In our recent laboratory report, we reported that CMC-EDBE-FA nanoparticle is nontoxic [27]. We also reported that in vivo challenge of VSSA and VRSA for five days can produce the highest degree of damage in lymphocytes through the increased production of nitric oxide, TNF- α that leads to decreased antioxidant status in cell and ten days successive treatment of nanoconjugated vancomycin also eliminate in vivo VSSA and VRSA infection [28]. Recently, we reported that, nanoconjugated vancomycin can be used as a potent free-radical scavenger antioxidative product and can be used as a potential therapeutic agent against staphylococcal infection [29]. The present study was aimed to test the protective role of nanoconjugated vancomycin against VSSA and VRSA induced oxidative imbalance in neutrophils.

2. Materials and Methods

2.1. Chemicals and Reagents. Histopaque 1077, dextran, Sodium dodecyl sulfates (SDS), 2,4-dinitrophenyl hydrazine (DNPH), 5',5'-dithio(bis)-2-nitrobenzoic acid (DTNB), standard reduced glutathione (GSH), glutathione reductase (GR), NADPH Na₄, NADPH, oxidized glutathione (GSSG), agarose, folic acid (FA), Chitosan (medium molecular weight), dicyclohexyl carbodiimide (DCC), Trifluoroacetic acid, 2, 2'-(ethylenedioxy)-bis-(ethylamine) (EDBE), di-tert-butylidicarbonate (BoC₂O), N-hydroxysuccinimide (NHS), and 1-[3-dimethylamino)propyl]-3-ethylcarbodiimide Hydrochloride (EDC) were purchased from Sigma Chemical Co., USA. Sodium chloride (NaCl), sodium dodecyl sulfate, ethylene diamine tetra acetate (EDTA), tryptic soy broth, luria broth, mannitol salt agar, agar powder, sucrose, magnesium chloride (MgCl₂), and sodium azide (NaN₃) were purchased from Himedia, India. Tris-Hcl, Trisbuffer, potassium dihydrogen phosphate (KH₂PO₄), dipotassium hydrogen phosphate (K₂HPO₄), sodium hydroxide (NaOH), sodium acetate, ammonium acetate, alcohol, sulfanilamide, phosphoric acid, and N-C-1 naphthyl ethylene diamine dihydrochloride and other chemicals were procured from Merck Ltd., SRL Pvt. Ltd., and Mumbai, India. All other chemicals were from Merck Ltd., SRL Pvt., Ltd., and 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 $25 \pm 2^\circ\text{C}$. 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. We used coagulase positive vancomycin sensitive (MMC-6) and resistant (MMC-17) *Staphylococcus aureus* strains that were isolated from human post-operative pus sample [30]. These bacterial strains were grown at 37°C for 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, we adjusted the viable bacterial count 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 phosphate buffer saline (PBS) to give a final concentration of approximately 5×10^6 in 100 μL of bacterial suspension [31].

2.4. Development of VSSA and VRSA Infection in Swiss Mice. VSSA and VRSA infection was developed in male Swiss mice by intraperitoneal (i.p.) injection of 100 μL of bacterial suspension containing 5×10^6 CFU/mL according to our previous laboratory report [28].

2.5. 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 [26].

2.6. Experimental Design. VSSA- and VRSA-infected mice were treated with nanoconjugated vancomycin for successive 10 days at a dose of 100 mg/kg bw/day and 500 mg/kg bw/day, respectively. The dose and duration of nanoconjugated vancomycin were selected from our previous laboratory report [28]. The following groups were considered for the experiment:

- Group I: control;
- Group II: VSSA-infected control;
- Group III: VSSA infection + 100 mg/kg bw/day vancomycin;
- Group IV: VSSA infection + 100 mg/kg bw/day nanoconjugated vancomycin;
- Group V: VRSA-infected control;
- Group VI: VRSA infection + 500 mg/kg bw/day vancomycin;

Group VII: VRSA infection + 500 mg/kg bw/day nanoconjugated vancomycin.

After the termination of experiment, animals were sacrificed by an intraperitoneal injection of sodium pentobarbital (60–70 mg/kg body weight) [32] and blood ($n = 6/\text{group}$) was used for preparation neutrophils for biochemical estimation of different oxidative parameters.

2.7. Separation of Neutrophils. Heparinized blood samples were used for the separation of neutrophils. Blood samples were diluted with equal amount of PBS (pH 7.0) buffer and then layered very carefully on the density gradient (Histo-paque 1077, Sigma Chemical Co.) in 1 : 2 ratio. Centrifuged at 500 g for 20 min and the white milky layer of mononuclear cells that is lymphocytes was carefully removed. Neutrophils were isolated from buffy coat with RBC layer followed by dextran sedimentation and hypotonic lysis to remove red blood cells. The pellets of neutrophil were lysed in a hypotonic lysis buffer for 45 min at 37°C and kept at –86°C until biochemical estimations of different parameters [33].

2.8. Biochemical Estimation

2.8.1. Nitrite (NO) Production by Neutrophils. NO generation in cell lysate was assessed according to Sanai et al. 1998, with slight modification [34]. Sodium nitroprusside (100 mM), in phosphate-buffered saline, was mixed with 200 μL sample and incubated at room temperature for 150 min. After that, Griess reagent (0.5 mL) (Containing 1% sulfanilamide in 5% phosphoric acid and 0.1% N-C-1 naphthyl ethylene diamine dihydrochloride in 1 : 1 ratio) was added and incubated at room temperature for 10 min. The absorbance of the chromophore formed was read at 550 nm with a double beam Hitachi U2001 UV/Visible spectrophotometer (USA). NO generation was calculated using the sodium nitrite standard curve and expressed as $\mu\text{mol}/\text{mg}$ protein.

2.8.2. Determination of Lipid Peroxidation (MDA) in Neutrophils. Lipid peroxidation was estimated by the method of Ohkawa et al. in cell lysate [35]. Briefly, the reaction mixture contained Tris-HCl buffer (50 mM, pH 7.4), tertbutyl hydroperoxide (BHP) (500 μM in ethanol) and 1 mM FeSO_4 . After incubating the samples at 37°C for 90 min, the reaction was stopped by adding 0.2 mL of 8% sodium dodecyl sulfate (SDS) followed by 1.5 mL of 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed during incubation was estimated by adding 1.5 mL of 0.8% TBA and further heating the mixture at 95°C for 45 min. After cooling, samples were centrifuged, and the TBA reactive substances (TBARS) were measured in supernatants at 532 nm by using $1.53 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ as extinction coefficient. The levels of lipid peroxidation were expressed in terms of $n \text{ mol}/\text{mg}$ protein.

2.8.3. Protein Carbonyls (PC) Contents in Neutrophils. Protein oxidation was monitored by measuring protein carbonyl contents by derivatization with 2,4-dinitrophenyl hydrazine

(DNPH) [36]. In general, cell lysate proteins in 50 mM potassium phosphate buffer, pH 7.4, were derivatized with DNPH (21% in 2 N HCl). Blank samples were mixed with 2 N HCl incubated at 1 h in the dark; protein was precipitated with 20% trichloro acetic acid (TCA). Underivatized proteins were washed with an ethanol: ethyl acetate mixture (1 : 1). Final pellets of protein were dissolved in 6.0 N guanidine hydrochloride and absorbance was measured at 370 nm. Protein carbonyls content was expressed in terms of $n \text{ mol}/\text{mg}$ protein.

2.8.4. Determination of Reduced Glutathione (GSH) Level in Neutrophils. Reduced glutathione estimation in the cell lysate was performed by the method of Moron et al. 1979 [37]. The required amount of the cell lysate was mixed with 25% of trichloroacetic acid and centrifuged at 2,000 g for 15 min to settle the precipitated proteins. The supernatant was aspirated and diluted to 1 mL with 0.2 M sodium phosphate buffer (pH 8.0). Later, 2 mL of 0.6 mM DTNB was added. After 10 min the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman's reagent) was measured at 405 nm. A standard curve was obtained with standard reduced glutathione. The levels of GSH were expressed as μg of GSH/mg protein.

2.8.5. Determination of Oxidized Glutathione (GSSG) Level in Neutrophils. The oxidized glutathione level was measured after derivatization of GSH with 2-vinylpyridine according to the method of Griffith, 1980 [38]. In brief, with 0.5 mL cell lysate, 2 μL 2-vinylpyridine was added and incubated for 1 hr at 37°C. Then the mixture was deproteinized with 4% sulfosalicylic acid and centrifuged at 1,000 g for 10 min to settle the precipitated proteins. The supernatant was aspirated and GSSG level was estimated with the reaction of DTNB at 412 nm in spectrophotometer and calculated with standard GSSG curve. The levels of GSSG were expressed as μg of GSSG/mg protein.

2.8.6. Activity of Super Oxide Dismutase (SOD) in Neutrophils. SOD activity was determined from its ability to inhibit the autooxidation of pyrogallol according to Mestro Del and McDonald, 1986 [39]. The reaction mixture considered is of 50 mM Tris (hydroxymethyl) aminomethane (pH 8.2), 1 mM diethylenetriamine penta acetic acid, and 20–50 μL of cell lysate. The reaction was initiated by addition of 0.2 mM pyrogallol, and the absorbance is measured kinetically at 420 nm at 25°C for 3 min. SOD activity was expressed as unit/mg protein.

2.8.7. Activity of Catalase (CAT) in Neutrophils. Catalase activity was measured in the cell lysate by the method of Luck, 1963 [40]. The final reaction volume of 3 mL contained 0.05 M tris-buffer, 5 mM EDTA (pH 7.0) and 10 mM H_2O_2 (in 0.1 M potassium phosphate buffer, pH 7.0). About 50 μL aliquot of the cell lysates was added to the above mixture. The rate of change of absorbance per min at 240 nm was recorded. Catalase activity was calculated by using the molar

extinction coefficient of $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ for H_2O_2 . CAT activity was expressed as unit/mg protein.

2.8.8. Activity of Glutathione Peroxidase (GPx) in Neutrophils. The GPx activity was measured by the method of Paglia and Valentine, 1967 [41]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 1 U glutathione reductase, and 1 mM reduced glutathione. The sample, after its addition, was allowed to equilibrate for 5 min at 25°C . The reaction was initiated by adding 0.1 mL of 2.5 mM H_2O_2 . Absorbance at 340 nm was recorded for 5 min. Values were expressed as n mol of NADPH oxidized to NADP by using the extinction coefficient of $6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 340 nm. The activity of GPx was expressed in terms of n mol NADPH consumed/min/mg protein.

2.8.9. Activity of Glutathione Reductase (GR) in Neutrophils. The GR activity was measured by the method of Miwa, 1972 [42]. The tubes for enzyme assay were incubated at 37°C and contained 2.0 mL of 9 mM GSSG, 0.02 mL of 12 mM NADPH, Na_4 , 2.68 mL of 1/15 M phosphate buffer (pH 6.6), and 0.1 mL of cell lysate. The activity of this enzyme was determined by monitoring the decrease in absorbance at 340 nm. The activity of GR was expressed in terms of n mol NADPH consumed/min/mg protein.

2.8.10. Activity of Glutathione-s-Transferase (GST) in Neutrophils. The activity of GST activity was measured by the method of Habig et al. 1974 [43]. The tubes of enzyme assay were incubated at 25°C and contained 2.85 mL of 0.1 M potassium phosphate (pH 6.5) containing 1 mM of GSH, 0.05 mL of 60 mM 1-chloro-2,4-dinitrobenzene, and 0.1 mL cell lysate. The activity of this enzyme was determined by monitoring the increase in absorbance at 340 nm. The activity of GST was expressed in terms of n mol NADPH consumed/min/mg protein.

2.8.11. Protein Estimation. Protein was determined using bovine serum albumin as standard according to Lowry et al. 1951 [44].

2.9. Statistical Analysis. The experiments were performed three times and the data are presented as mean \pm S.E.M. 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. Nitrate (NO) Generation. Nitrite generation (NO) that occurred by inducible nitric oxide synthase (iNOS) can then combine with superoxide and be able to generate a product which has much more toxicity such as peroxynitrite (ONOO^-). NO generation was significantly ($P < 0.05$) increased in VSSA- and VRSA-infected neutrophils by 135.45%

and 145.32%, respectively in compare with control, which was also significantly ($P < 0.05$) decreased in nanoconjugated vancomycin-treated group by 46.30% and 49.44%. It was observed from our study that treatment of vancomycin significantly ($P < 0.05$) decreased the NO generation by 32.68% in VSSA infection, whereas 5.64% in VRSA infection, which was not significant (Figure 1).

3.2. Lipid Peroxidation Level. Lipid peroxidation is an important determinant to assess the cellular damage. Lipid peroxidation in terms of malondialdehyde level was significantly ($P < 0.05$) increased in neutrophils by 145.28% and 186.48%, respectively, due to VSSA and VRSA infection as compared to control group, which was significantly ($P < 0.05$) decreased by 44.52% and 45.37%, respectively, due to treatment of nanoconjugated vancomycin. Treatment of vancomycin decreased MDA levels significantly ($P < 0.05$) in VSSA-infected neutrophils by 34.39%, whereas 4.59% in VRSA-infected neutrophils which was not significant (Figure 2).

3.3. Protein Oxidation Level. Protein oxidation in terms of protein carbonyls (PC) is an important determination of cellular injury. Protein carbonyl level was significantly ($P < 0.05$) increased in neutrophils by 140.38% and 187.44%, respectively, due to VSSA and VRSA infection as compared to control group, which was significantly ($P < 0.05$) decreased by 45.19% and 46.94%, respectively, due to treatment of nanoconjugated vancomycin. Treatment of vancomycin decreased PC levels significantly ($P < 0.05$) in VSSA-infected neutrophils by 33.81%, whereas 10.89% in VRSA-infected neutrophils which was not significant (Figure 2).

3.4. Reduced and Oxidized Glutathione Level. Glutathione is an important antioxidant in cellular system. So, to understand glutathione level, we have measured both reduced and oxidized form of glutathione. Reduced glutathione level was significantly ($P < 0.05$) diminished in neutrophils by 67.50% and 53.04%, respectively, due to VSSA and VRSA infection as compared to control group, which was significantly ($P < 0.05$) increased by 161.62% and 91.98% due to treatment of nanoconjugated vancomycin. Treatment of vancomycin increased reduced glutathione level significantly ($P < 0.05$) in VSSA-infected neutrophils by 128.14%, whereas 3.84% in VRSA-infected neutrophils which is not significant (Figure 3). Oxidized glutathione level was significantly ($P < 0.05$) elevated in neutrophils by 90.11% and 103.64%, respectively, due to VSSA and VRSA infection as compared to control group, which was significantly ($P < 0.05$) decreased by 40.64% and 38.43% due to treatment of nanoconjugated vancomycin. Treatment of vancomycin decreased oxidized glutathione level significantly ($P < 0.05$) in VSSA-infected neutrophils by 23.01%, whereas 5.15% in VRSA-infected neutrophils which was not significant (Figure 3).

3.5. Superoxide Dismutase (SOD) Activity. Superoxide dismutase catalyzes the breakdown of superoxide radical into

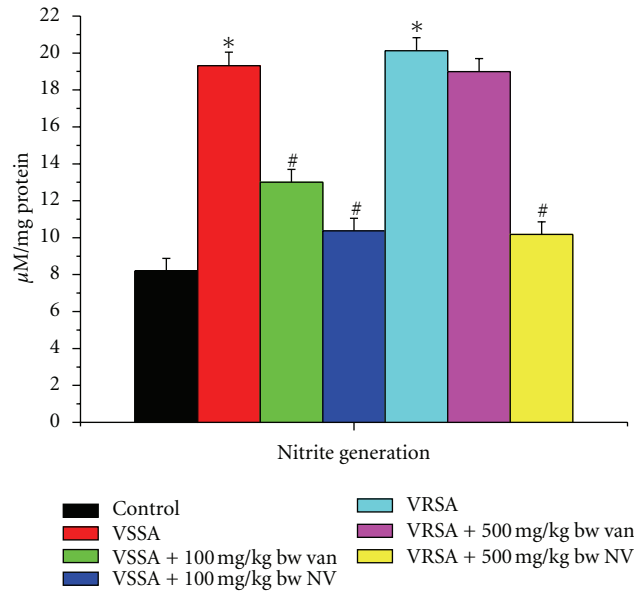


FIGURE 1: Nitrite generation (NO) in control, VSSA infection control, VSSA infection + 100 mg/kg bw/day vancomycin, VSSA infection + 100 mg/kg bw/day nanoconjugated vancomycin, VRSA infection control, VRSA infection + 500 mg/kg bw/day vancomycin, and VRSA infection + 500 mg/kg bw/day nanoconjugated vancomycin for 10 days treated neutrophils. Values are expressed as mean ± SEM, *n* = 6. *Indicates significant difference (*P* < 0.05) compared to control group. #Indicates significant difference (*P* < 0.05) compared to infection control group.

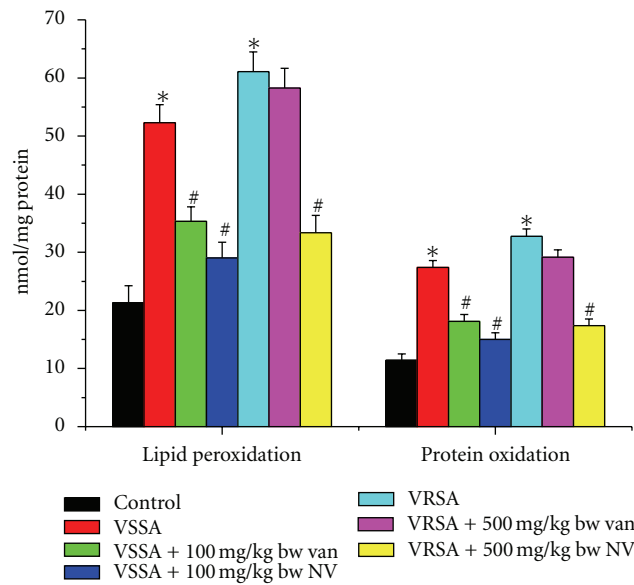


FIGURE 2: Lipid peroxidation (MDA) and protein oxidation (PC) level in control, VSSA infection control, VSSA infection + 100 mg/kg bw/day vancomycin, VSSA infection + 100 mg/kg bw/day nanoconjugated vancomycin, VRSA infection control, VRSA infection + 500 mg/kg bw/day vancomycin, and VRSA infection + 500 mg/kg bw/day nanoconjugated vancomycin for 10 days treated neutrophils. Values are expressed as mean ± SEM, *n* = 6. *Indicates significant difference (*P* < 0.05) compared to control group. #Indicates significant difference (*P* < 0.05) compared to infection control group.

oxygen and hydrogen peroxide. SOD activity was significantly (*P* < 0.05) diminished in neutrophils by 44.03%, and 35.31%, respectively, due to VSSA and VRSA infection as compared to control group, which was significantly (*P* < 0.05) increased by 67.16% and 40.09% due to treat-

ment of nanoconjugated vancomycin. Treatment of vancomycin increased SOD activity significantly (*P* < 0.05) in VSSA-infected neutrophils by 39.24%, whereas 11.25% in VRSA-infected neutrophils which was not significant (Figure 4).

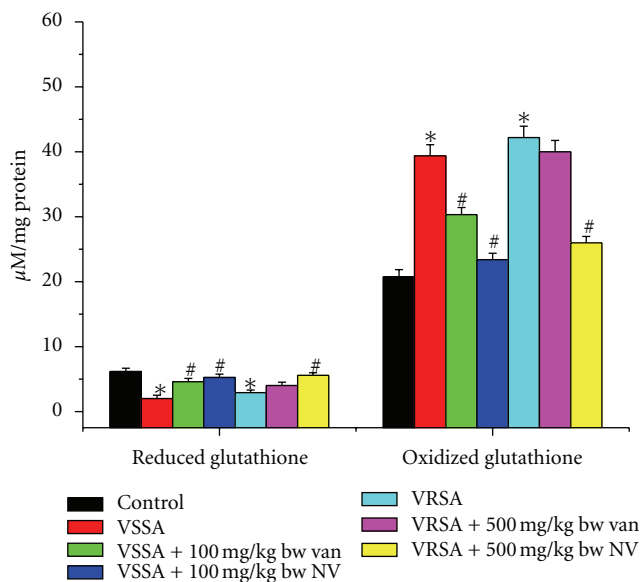


FIGURE 3: Reduced glutathione (GSH) and oxidized glutathione (GSSG) level in control, VSSA infection control, VSSA infection + 100 mg/kg bw/day vancomycin, VSSA infection + 100 mg/kg bw/day nanoconjugated vancomycin, VRSA infection control, VRSA infection + 500 mg/kg bw/day vancomycin, and VRSA infection + 500 mg/kg bw/day nanoconjugated vancomycin for 10 days treated neutrophils. Values are expressed as mean \pm SEM, $n = 6$. * Indicates significant difference ($P < 0.05$) compared to control group. # Indicates significant difference ($P < 0.05$) compared to infection control group.

3.6. Catalase (CAT) Activity. Catalase catalyzes the conversion of hydrogen peroxide to oxygen and water. CAT activity was significantly ($P < 0.05$) decreased in neutrophils by 61.40% and 56.47%, respectively, due to VSSA and VRSA infection as compared to control group, which was significantly ($P < 0.05$) increased by 99.44% and 123.04% due to treatment of nanoconjugated vancomycin. Treatment of vancomycin increased CAT activity significantly ($P < 0.05$) in VSSA-infected neutrophils by 24.99%, whereas 1.36% in VRSA-infected neutrophils which was not significant (Figure 4).

3.7. Glutathione Peroxidase (GPx) Activity. The glutathione peroxidase (GPx) activity was measured to understand the antioxidant enzyme status of different experimental group of neutrophils. GPx activity was significantly ($P < 0.05$) diminished in neutrophils by 74.17% and 75.70%, respectively, due to VSSA and VRSA infection as compared to control group, which was significantly ($P < 0.05$) increased by 217.13% and 248.95% due to treatment of nanoconjugated vancomycin. Treatment of vancomycin increased GPx activity significantly ($P < 0.05$) in VSSA-infected neutrophils by 155.94%, whereas 8.42% in VRSA-infected neutrophils which was not significant (Figure 5).

3.8. Glutathione Reductase (GR) Activity. The glutathione reductase (GR) activity was measured to understand the antioxidant enzyme status of different experimental group of neutrophils. GR activity was significantly ($P < 0.05$) decreased in neutrophils by 56.17% and 57.19%, respectively, due to VSSA and VRSA infection as compared to control group, which was significantly ($P < 0.05$) increased by

104.65% and 111.96% due to treatment of nanoconjugated vancomycin. Treatment of vancomycin increased GR activity significantly ($P < 0.05$) in VSSA-infected neutrophils by 58.64%, whereas 4.74% in VRSA-infected neutrophils which was not significant (Figure 5).

3.9. Glutathione-s-Transferase (GST) Activity. The glutathione-s-transferase (GST) activity was measured to understand the antioxidant enzyme status of different experimental group of neutrophils. GST activity was significantly ($P < 0.05$) diminished in neutrophils by 50.13% and 50.62%, respectively, due to VSSA and VRSA infection as compared to control group, which was significantly ($P < 0.05$) increased by 73.62% and 78.38% due to treatment of nanoconjugated vancomycin. Treatment of vancomycin increased GST activity significantly ($P < 0.05$) in VSSA-infected neutrophils by 48.0%, whereas 6.35% in VRSA-infected neutrophils which was not significant (Figure 5).

4. Discussion

Nature has provided cells with very strong biological antioxidant defense mechanisms. These include a variety of enzymatic and nonenzymatic molecules with enormous capabilities to mitigate the deleterious and potentially harmful effects of ROS and other free radicals. One of the primary antioxidant defense mechanisms is the GSH redox system. The enzymes of this system provide a formidable protective shield against oxidative damage. Alterations in their activities ultimately may result in irreversible manifestation of cellular damage [45]. In this context, our present study proves to be more relevant and will help further study in investigating the

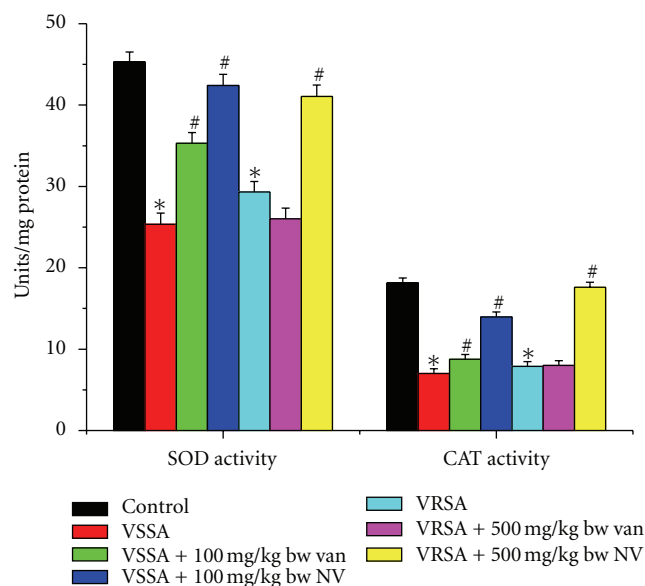


FIGURE 4: Activity of superoxide dismutase (SOD) and Catalase (CAT) in control, VSSA infection control, VSSA infection + 100 mg/kg bw/day vancomycin, VSSA infection + 100 mg/kg bw/day nanoconjugated vancomycin, VRSA infection control, VRSA infection + 500 mg/kg bw/day vancomycin, and VRSA infection + 500 mg/kg bw/day nanoconjugated vancomycin for 10 days treated neutrophils. Values are expressed as mean \pm SEM, $n = 6$. *Indicates significant difference ($P < 0.05$) compared to control group. #Indicates significant difference ($P < 0.05$) compared to infection control group.

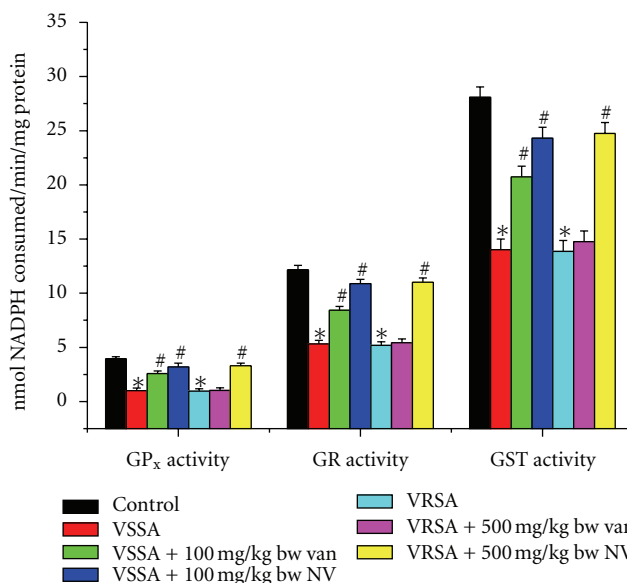


FIGURE 5: Activity glutathione peroxidase (GP_x), glutathione reductase (GR) and glutathione-s-transferase (GST) in control, VSSA infection control, VSSA infection + 100 mg/kg bw/day vancomycin, VSSA infection + 100 mg/kg bw/day nanoconjugated vancomycin, VRSA infection control, VRSA infection + 500 mg/kg bw/day vancomycin, and VRSA infection + 500 mg/kg bw/day nanoconjugated vancomycin for 10 days treated neutrophils. Values are expressed as mean \pm SEM, $n = 6$. *Indicates significant difference ($P < 0.05$) compared to control group. #Indicates significant difference ($P < 0.05$) compared to infection control group.

protective role of nanoconjugated vancomycin against VSSA- and VRSA-induced oxidative stress in neutrophils.

CMC-EDBE-FA nanoparticle was prepared by the carboxylic group ($-\text{COOH}$) of folic acid and $-\text{COOH}$ group of functionalized carboxymethyl chitosan connected through the end-amino groups hydrophilic spacer, 2,2'-(ethylenedi-

oxy)-bis-ethylamine. It is well known that carboxymethyl chitosan is easily soluble in water but folic acid is very less solubility in water. When carboxymethyl chitosan is connected by folic acid through a spacer, carboxymethyl chitosan may act as a hydrophilic part and folic acid as a hydrophobic part. It is evident from our study that, in vivo VSSA and VRSA

infection in neutrophils of mice is associated with enhanced nitrate generation, MDA level, PC level, GSSG level, and decreased GSH level and as well as decreased enzymatic antioxidant (SOD, CAT, GPx, GR, and GST) activity, which are ameliorated by treatment of nanoconjugated vancomycin (Figures 1–5).

In this study, significant elevation of nitrate generation in neutrophils was observed in VSSA- and VRSA-infected mice. Treatment of nanoconjugated vancomycin to VSSA- and VRSA-infected mice decreased the NO generation (Figure 1). Nitric oxide (NO) is a free radical synthesized by nitric oxide synthase (NOS). NOS is composed of two identical monomers with molecular weights ranging from 130 to 160 kDa [46]. Our previous study shown that nitric oxide synthesis in lymphocytes and as well as release in serum is high during VSSA and VRSA infection, which can be related to an alteration in oxidant-antioxidant potential [28]. Thus, higher level of nitrite generation by VSSA and VRSA infection may be due to high production of ROS. Nanoconjugated vancomycin plays the role of antioxidant to prevent the nitrate generation may be through the inhibition of inducible nitric oxide synthase (iNOS) expression [47]. Thus, in addition to the cellular antioxidant system, nanoconjugated vancomycin may indirectly protect neutrophils from VSSA and VRSA infection induced cellular changes. Thus, free radical depletion by the antioxidant agents seems to be beneficial for preventing the damage of lipid and protein.

In this study, significant elevation of malondialdehyde (MDA) and protein carbonyl level was observed in neutrophils of VSSA- and VRSA-infected mice. Treatment of nanoconjugated vancomycin to VSSA- and VRSA-infected mice decreased lipid peroxidation and protein oxidation significantly in neutrophils (Figure 2). It may be due to the generation of free radicals (mainly NO) which may react with protein in addition to lipids. Lipid peroxidation is known to disturb the integrity of cellular membranes leading to the leakage of cytoplasmic enzymes [48]. 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 cleanup oxidative damaged proteins. Oxidative modification of proteins may lead to the structural alteration and functional inactivation of many enzyme proteins [49], as evidenced by the decreased activity of different antioxidant enzymes like SOD, CAT, GPx, GR, and GST.

Reactive oxygen species (ROS) are generated during oxidative metabolism and can inflict damage on all classes of cellular macromolecules and eventually leading to cell death. An elevation in free radical formation can be accompanied by an immediate compensatory increase in the activities of the free radical scavenging enzymes [50]. Imbalance between the generation of reactive oxygen species (ROS) and the antioxidant system causes oxidative stress. Glutathione, an important cellular reductant, is involved in protection against free radicals, peroxides, and toxic compounds in cellular systems [51]. In the present study, the reduced glutathione level was significantly decreased in neutrophils of VSSA- and VRSA-infected mice. Treatment of nanoconjugated vancomycin to VSSA- and VRSA-infected mice increased the GSH level

(Figure 3). In this study, it was observed that oxidized glutathione level was increased in VSSA- and VRSA-infected neutrophils, which was ameliorated due to nanoconjugated vancomycin treatment (Figure 3). The decreased GSH levels represent its increased utilization due to VSSA and VRSA infection. On the other hand, decreasing GSH level may be due to increasing level of lipid oxidation products which may be associated with less availability of NADPH required for the activity of glutathione reductase (GR) to transform GSSG to GSH [52] due to the increasing production of ROS in form of NO. In our present study, the increasing levels of GSSG and decreasing GR activity (Figure 5) due to VSSA and VRSA infection may support the explanation.

Antioxidant enzymes are considered to be a primary defense that prevents biological macromolecules from oxidative damage. SOD rapidly dismutates superoxide anion ($O_2^{\bullet-}$) to less dangerous H_2O_2 , which is further degraded by CAT and GPx to water and oxygen [53]. The results of the present study showed a significant fall in SOD and CAT activities in neutrophils of VSSA- and VRSA-infected group. Treatment of nanoconjugated vancomycin to VSSA- and VRSA-infected mice significantly increased the SOD and CAT activity in neutrophils (Figure 4). SOD, dismutate $O_2^{\bullet-}$ and the same in turn is a potent inhibitor of CAT [54]. The depletion in SOD activity may be due to dispose off the free radicals, produced due to VSSA and VRSA infection. Beside this, during infection, H_2O_2 produced by dismutation of superoxide anion may have been efficiently converted to O_2 by CAT and the enzyme activities showed a marked reduction. The depletion of antioxidant enzyme activity may be due to inactivation of the enzyme proteins by VSSA and VRSA infection induced NO generation, depletion of the enzyme substrates, and/or downregulation of transcription and translation processes.

GPx works nonspecifically to scavenge and decompose excess hydroperoxides including H_2O_2 , which may be prevalent under oxidative stress [55–57]. Glutathione-s-transferase (GST) mainly detoxifies electrophilic compounds [58] and has a well-established role in protecting cells from mutagens and carcinogens as a free-radical scavenger along with glutathione. In the present study, the significant decreasing of GSH level and GSH-dependent enzymes, that is, GPx, GR, and GST (Figure 5) in neutrophils of VSSA and VRSA infection may be due to increased utilization to scavenge the free-radical generation. Treatment of nanoconjugated vancomycin to VSSA- and VRSA-infected mice significantly increased the GPx, GR, and GST activity in neutrophils (Figure 5).

In conclusion, the study described here, neutrophils are susceptible to *S. aureus* infection through the increased production of nitric oxide which leads to decreased antioxidant status in cell and nanoconjugated vancomycin protects the neutrophils from such infection by decreasing NO generation, lipid, and protein damage and also by increasing the antioxidant status. Hence, the nanoconjugated vancomycin may be used as a potent free-radical scavenger antioxidative product and as well as a potential therapeutic agent against staphylococcal infection.

Abbreviations

CAT:	Catalase
CFU:	Colony formation unit
CMC:	Carboxymethyl chitosan
CMC-EDBE-FA:	Carboxymethyl chitosan-2,2' ethylenedioxy bis ethylamine-Folate
CS:	Chitosan
DTNB:	5',5'-dithio(bis)-2-nitrobenzoic acid
EDBE:	2, 2' ethylenedioxy bis-ethylamine
EDTA:	Ethylene diamine tetra acetate
GPx:	Glutathione peroxidase
GR:	Glutathione reductase
GSH:	Reduced glutathione
GSSG:	Oxidized glutathione
GST:	Glutathione-s-transferase
ip:	Intraperitoneal
H ₂ O ₂ :	Hydrogen peroxide
iNOS:	Inducible nitric oxide synthase
MDA:	Malondialdehyde
NO:	Nitric oxide
PBS:	Phosphate buffer saline
PMN:	Polymorphonuclear neutrophils
ROS:	Reactive oxygen species
rpm:	Rotation per minute
<i>S. aureus</i> :	<i>Staphylococcus aureus</i>
SDS:	Sodium dodecyl sulfate
SOD:	Superoxide dismutase
SSA:	Sulfosalicylic acid
TBARS:	Thiobarbituric acid reactive substance
TCA:	Trichloro acetic acid
VRSA:	Vancomycin resistant <i>Staphylococcus aureus</i> .

Conflict of Interests

The authors declare that there is no conflict of interests.

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