VANCOMYCIN RESISTANT STAPHYLOCOCCUS AUREUS INDUCED OXIDATIVE STRESS IN LIVER AND KIDNEY: PROTECTIVE ROLE OF NANOCONJUGATED VANCOMYCIN

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ABSTRACT

Staphylococcus aureus is a most common pathogen in hospital and community acquired disease that causes a wide range of infection such as skin and soft tissue infection to life threatening disease like respiratory tract infection, musculoskeletal infection, endocarditis and urinary tract infection. The aim of the present study was to evaluate the possible protective effects of nanoconjugated vancomycin against VRSA infection on selective makers of oxidative damage and antioxidant status in liver and kidney. A coagulase positive VRSA strain was used for this study. VRSA infection was developed in Swiss mice by intraperitoneal injection of $5 \times 10^6$ CFU/ml bacterial solutions. Nanoconjugated vancomycin was treated to VRSA infected mice at its effective dose for 10 days. After decapitation, liver and kidney were excised from control and experimental groups, homogenized and used for different biochemical estimation. Nitrate level, myeloperoxidase activity, lipid peroxidation, protein oxidation, oxidized glutathione, DNA fragmentation level were increased significantly ($p<0.05$) in liver and kidney of VRSA infected group as compared to control group, and reduced glutathione level, activity of antioxidant enzymes (SOD and CAT), glutathione dependent enzymes (GPx, GR and GST) were decreased significantly ($p<0.05$); which were increased or decreased significantly ($p<0.05$) near to normal in nanoconjugated vancomycin treated group. These finding suggests the potential use and beneficial role of nanoconjugated vancomycin against VRSA infection induced oxidative stress and DNA damage in liver and kidney.

Key-words: VRSA, free radical, lipid peroxidation, antioxidant enzyme, DNA fragmentation.

1. INTRODUCTION

Staphylococcus aureus is facultatatively anaerobic, gram positive coccus and is the most common cause of staph infections (Kluytmans J et al. 1997). S. aureus has developed resistance to most classes of antimicrobial agents. Penicillin was the first choice of antibiotics to treat staphylococcal infection. In 1944, by destroying the penicillin by penicillinase, S. aureus become resistant (Kirby W. 1944). More than 90% S. aureus strains are resistant to penicillin (Neu H. 1992). Methicillin, a semi synthetic penicillin was used to treat Penicillin Resistant Staphylococcus aureus but resistance finally emerge in 1962.
Vancomycin, a glycopeptide antibiotic continues to be an important antimicrobial agent to treat MRSA but resistance finally emerges. In June 2002, the World’s first reported clinical infection due to S. aureus with high resistance to vancomycin (VRSA) was diagnosed in a patient in the USA (Sievert D et al. 2002). Recently, we have isolated thirty pathogenic S. aureus from post operative pus sample by standard biochemical test and detection of S. aureus specific nuc gene; out of them twenty two were vancomycin sensitive and rests were vancomycin resistant (Chakraborty S et al. 2011c).

S. aureus causes chronic/relapsing diseases and reported to persist as an opportunistic intracellular organism both in vitro and in vivo (Brouillette E et al. 2003). S. aureus were able to survive within phagocytic cells both in polymorphonuclear leukocytes (PMN) and monocytes (Steigbigel R et al. 1974). In vitro studies 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. To induce an infection in host the pathogenic must cope with their changing environment and have to attack continuously to the host to weak the immune system (Dryla A et al. 2003).

2 MATERIALS AND METHODS

2.1 Chemicals and reagents
Sodium dodecyl sulfate (SDS), 5', 5'-dithio (bis)-2-nitrobenzoic acid (DTNB), standard reduced glutathione (GSH), glutathione reductase (GR), NADPH, Na₂, NADPH, oxidized glutathione (GSSG) were purchased from Sigma Chemical Co., USA. Sodium chloride (NaCl), sodium dodecyl sulfate, sucrose, ethylene diamine tetra acetate (EDTA), tryptic soy broth, mannitol salt agar, agarose was purchased from Himedia, India. Tris-HCl, KH₂PO₄ and K₂HPO₄, alcohol, diphenylamine (DPA), O-phenylenediamine 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 eighteen (18) 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 °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 a coagulase positive Vancomycin resistant *Staphylococcus aureus* (MMC-17) strain that was isolated from human post operative pus sample and was grown at 37°C for overnight in tryptic soy broth (Chakraborty S et al. 2011c). The bacterial culture was centrifuged at 15,000 rpm for 15 minutes. 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 $1.0 \times 10^9$ 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 give a final concentration of approximately $5 \times 10^6$ in 100µl of bacterial suspension (Hattie D et al. 2000).

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 (Chakraborty S et al. 2010).

2.5 Development of VRSA infection in Swiss mice
VRSA infection was developed in male Swiss mice by intraperitoneal (i.p.) injection of 100µl of bacterial suspension containing $5 \times 10^6$ CFU/ml according to our previous laboratory report (Chakraborty S et al. 2011b).

2.6 Experimental design
VRSA infected mice were treated with nanoconjugated vancomycin for successive 10 days at a dose of 500 mg/kg bw/day. The dose and duration of nanoconjugated vancomycin was selected from our previous laboratory report (Chakraborty S et al. 2011b). The following groups were considered for the experiment: **Group I**: Control, **Group II**: VRSA infection, **Group III**: VRSA infection + 500 mg/kg bw/day nanoconjugated vancomycin. After the termination of experiment, animals were sacrificed by an intraperitoneal injection of sodium pentobarbitral (60–70 mg/kg body weight) (Chandran K and Venugopal PM, 2004).

2.7 Separation and homogenization of liver and kidney
After decapitation, liver and kidney were excised from experimental mice of different experimental groups and washed with cold normal saline and homogenized in the ice-cold buffer containing 0.25 M sucrose, 1 mM EDTA, and 1 mM Tris-Hcl, pH 7.4. The homogenate was first centrifuge at 600×g for 10 min at 4°C, and the supernatant was stored at -80ºC for the biochemical estimation of different parameters.

2.8 Biochemical estimation
2.8.1 Nitrite (NO) level
After treatment schedule, 100 μl of Griess reagent (containing 1 part of 1% sulfanilamide in 5% phosphoric acid, and 1 part of 0.1% of N-C-1 naphthyl ethylene diamine dihydrochloride) was added to 100 μl of sample, incubated at room temperature for 10 minutes, readings were taken in a UV spectrophotometer at 550nm and compared to a sodium nitrite standard curve (values ranging between 0.5 and 25μM). The levels of NO were expressed as μM/mg protein (Chakraborty S et al. 2011b).

2.8.2 Determination of myeloperoxidase (MPO) activity
200 µl of sample was reacted with 200 µl substrate (containing H$_2$O$_2$ and OPD) in dark for 30 min. The blank was prepared with citrate phosphate buffer (pH 5.2) and substrate, in absence of cell free supernatant. The reaction was stopped with addition of 100 µl 2(N) sulfuric acid and reading was taken at 492 nm in a spectrophotometer (Chakraborty S et al. 2011b). The MPO activity was expressed in terms of μ M/mg protein.
2.8.3 Determination of Lipid Peroxidation (MDA) level
Lipid peroxidation of liver and kidney homogenate was estimated by the method of KarMahapatra et al., 2009 (Kar Mahapatra S et al. 2009). Briefly, the reaction mixture contained Tris-HCl buffer (50 mM, pH 7.4), tetra-butyl hydroperoxide (BHP) (500 μM in ethanol) and 1 mM FeSO4. 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 x 10^{5} M^{-1} cm^{-1} as extinction coefficient. The levels of lipid peroxidation were expressed in terms of n mol/mg protein.

2.8.4 Determination of protein carbonyls (PC) contents
Protein oxidation was monitored by measuring protein carbonyl contents by derivatization with 2, 4-dinitrophenyl hydrazine (DNPH) (Kar Mahapatra S et al. 2009). In general, liver and kidney proteins in 50mM potassium phosphate buffer, pH 7.4, were derivatized with DNPH (21% in 2N HCl). Blank samples were mixed with 2N HCl incubated at 1h 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 6N guanidine hydrochloride and absorbance was measured at 370nm. Protein carbonyls content was expressed in terms of n mol/mg protein.

2.8.5 Determination of reduced glutathione (GSH) level
Reduced glutathione estimation in liver and kidney homogenate was performed by the method of KarMahapatra et al., 2009 (Kar Mahapatra S et al. 2009). The required amount of sample was mixed with 25% of TCA 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 minutes 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.

2.8.6 Determination of oxidized glutathione (GSSG) level
The oxidized glutathione level in liver and kidney homogenate was measured after derevatization of GSH with 2-vinylpyridine according to the method of KarMahapatra et al., 2009 (Kar Mahapatra S et al. 2009). In brief, with 0.5 ml sample, 2 μl of 2-vinylpyridine was added and incubates 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.

2.8.7 Determination of super oxide dismutase (SOD) activity
SOD activity of liver and kidney homogenate was determined from its ability to inhibit the auto-oxidation of pyrogalol according to KarMahapatra et al., 2009 (Kar Mahapatra S et al. 2009). The reaction mixture considered of 50 mM Tris (hydroxymethyl) amino methane (pH 8.2), 1 mM diethylenetriamine penta acetic acid, and 20–50 μl of sample. The reaction was initiated by addition of 0.2 mM pyrogalol, and the absorbance measured kinetically at 420 nm at 25°C for 3 min. SOD activity was expressed as unit/ mg protein.

2.8.8 Determination of catalase (CAT) activity
Catalase activity of liver and kidney homogenate was measured by the method of KarMahapatra et al., 2009 (Kar Mahapatra S et al. 2009). The final reaction volume of 3 ml contained 0.05 M Tris-buffer, 5 mM EDTA (pH 7.0), and 10 mM H₂O₂ (in 0.1 M
potassium phosphate buffer, pH 7.0). About 50 µl of sample 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 M⁻¹ cm⁻¹ for H₂O₂. The level of CAT was expressed as unit/ mg protein.

2.8.9 Determination of glutathione peroxidase (GPx) activity
The GPx activity of liver and kidney homogenate was measured by the method of Kar Mahapatra et al., 2009 (Kar Mahapatra S et al. 2009). 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 H₂O₂. Absorbance at 340 nm was recorded for 5 min. Values were expressed as n mol of NAPDH oxidized to NADP by using the extinction coefficient of 6.2 x 10³ M⁻¹ cm⁻¹ at 340 nm. The activity of GPx was expressed in terms of n mol NADPH consumed/min/mg protein.

2.8.10 Determination of glutathione reductase (GR) activity
The GR activity liver and kidney homogenate was measured by the method of Kar Mahapatra et al., 2009 (Kar Mahapatra S et al. 2009). 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, Na₄, 2.68 ml of 1/15 M phosphate buffer (pH 6.6) and 0.1 ml of sample. 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.

2.8.11 Determination of glutathione-s-transferase (GST) activity
The GST activity of liver and kidney homogenate was measured by the method of Kar Mahapatra et al., 2009 (Kar Mahapatra S et al. 2009). 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 of sample. 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.

4.8.12 DNA fragmentation assay by diphenylamine (DPA) assay
The diphenylamine (DPA) reaction of liver and kidney was performed by the method of Paradones et al., 1993 (Paradones C et al. 1993). Perchloric acid (0.5 M) was added to the sample containing uncut DNA (resuspended in 200 µl of hypotonic lysis buffer) and to the other half of the supernatant containing DNA fragments. Then two volumes of a solution consisting of 0.088 M DPA, 98% (v/v) glacial acetic acid, 1.5% (v/v) sulphuric acid, and a 0.5% (v/v) concentration of 1.6% acetaldehyde solution were added. The samples were stored at 4°C for 48 h. The reaction was quantified spectrophotometrically at 575 nm. The percentage of fragmentation was calculated as the ratio of DNA in the supernatants to the total DNA.

2.8.13 Protein estimation
Protein was determined using bovine serum albumin as standard according to Lowry et al. 1951 (Lowry O et al. 1951).

2.9 Statistical analysis
The data were expressed as mean ± SEM, n=6. Comparisons between the means of control and VRSA treated group 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 Nitrite (NO) level and Myeloperoxidase (MPO) activity
Nitrate (NO) is an indicator of free radical generation. Myeloperoxidase (MPO) is an important enzyme to produce hypochlorus acid (HOCl) in cellular system that leads to oxidative damage. NO level and MPO activity were significantly (P < 0.05) increased by 251.05%, 139.68% and 156.45%, 122.18% in VRSA
infected liver and kidney, respectively, as compared to control group, which were significantly \( P < 0.05 \) decreased by 49.56\%, 49.14\% and 45.52\%, 45.95\%, respectively, due to treatment of nanoconjugated vancomycin (Figure 1-2).

![Figure 1: Nitrite (NO) generation in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. 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 VRSA-infected group.]

![Figure 2: Myeloperoxidase (MPO) activity in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. 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 VRSA-infected group.]

3.2 Lipid peroxidation (MDA) and protein oxidation (PC) level
Lipid peroxidation and protein oxidation are two important determinants to access the cellular damage. Lipid peroxidation in terms of malondialdehyde level and protein oxidation in terms of protein carbonyl level were significantly \( P < 0.05 \) increased by 270.20\%, 125.55\% and 222.39\%, 110.09\% in VRSA infected liver and kidney, respectively, as compared to control group, which were significantly \( P < 0.05 \) decreased by 61.19\%, 42.07\% and 55.86\%, 40.01\%, respectively, due to treatment of nanoconjugated vancomycin (Figure 3-4).
Figure 3: Lipid peroxidation (MDA) level in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. 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 VRSA-infected group.

Figure 4: Protein carbonyls (PC) contents in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. 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 VRSA-infected group.

3.3 Reduced glutathione (GSH) and oxidized glutathione (GSSG)
Glutathione is an important antioxidant in cellular system. So, to understand glutathione level, we have measured both reduced and oxidized form of glutathione. The reduced glutathione level was decreased significantly (P < 0.05) by 59.26% and 54.65% in liver and kidney of VRSA infected group, respectively, as compared to control; whereas the oxidized glutathione level was increased significantly (P <0.05) by 109.41% and 106.97%, respectively, as compared to control. Treatment of nanoconjugated vancomycin significantly (P <0.05) increased GSH level by 116.16% and 99.55%; and decreased GSSG level significantly (P <0.05) by 30.48% and 42.06%, respectively, in liver and kidney (Fig. 5-6).
3.4 Superoxide dismutase (SOD) and catalase (CAT) activity
The super oxide dismutase (SOD) and catalase (CAT) activity were measured to understand the antioxidant enzymes status in liver and kidney of VRSA infected group. SOD and CAT activity were decreased significantly (P<0.05) by 44.71%, 61.48% and 51.40%, 61.99% in VRSA infected liver and kidney, respectively, as compared to control group, which were significantly (P < 0.05) increased by 55.71%, 129.08% and 78.80%, 133.42%, respectively, due to treatment of nanoconjugated vancomycin (Figure 7-8).
3.5 Glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-s-transferase (GST) activity

The activity of glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-s-transferase (GST) were measured to understand the antioxidant enzymes status in liver and kidney of VRSA infected group. GPx, GR and GST activity were decreased significantly (P<0.05) by 75.47%, 76.33%; 57.30%, 61.73% and 59.35%, 66.13% in VRSA infected liver and kidney, respectively, as compared to control group, which were significantly (P < 0.05) increased by 267.93%, 286.54%; 123.08%, 140.39% and 120.94%, 163.61%, respectively, due to treatment of nanoconjugated vancomycin (Figure 9-11).
Figure 9: Glutathione peroxidase (GPx) activity in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. 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 VRSA-infected group.

Figure 10: Glutathione reductase (GR) activity in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. 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 VRSA-infected group.

Figure 11: Glutathione-s-Transferase (GST) activity in liver and kidney of control, VRSA-infected and nanoconjugated vancomycin treated group. 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 VRSA-infected group.
3.6 DNA fragmentation
DNA fragmentation was significantly \((P < 0.05)\) increased in liver and kidney by 50.66% and 62.98% as compared to control which was significantly \((P < 0.05)\) decreased by 63.70% and 72.40% due to treatment of nanoconjugated vancomycin (Figure 12).

4. DISCUSSION

Staphylococcal infections caused by VRSA are increasing and their reduced therapeutic responsiveness to vancomycin represents an emerging threat to public health. Infection is a major complication of implanted devices. Patients frequently do not respond to high doses of antimicrobial agents even if administered for prolonged periods, and foreign bodies usually must be removed to achieve cure. It is known, susceptibility to antibiotics may be profoundly affected by growth of microorganisms near biomaterials. In vivo models are characterized by an artificial environment in which bacteria grow for a short time in the absence of host factors under selected, not necessarily physiologic, conditions.

The result of our study demonstrate that, VRSA infection in mice is associated with enhanced nitrate level, MPO activity, MDA level, PC level, GSSG level and decreased GSH level and as well as decreased enzymatic antioxidant (SOD, CAT, GPx, GR and GST) activity in liver and kidney, which are ameliorated by treatment of nanoconjugated vancomycin (Figure 1-11). Moreover DNA damage assessed by DPA assay due to VRSA infection was also observed in liver and kidney, which is protected by treatment of nanoconjugated vancomycin (Figure 12).

In this study, significant elevation of nitrate level and MPO activity in liver and kidney was observed in VRSA infected mice; which were decreased in nanoconjugated vancomycin treated group. Treatment of nanoconjugated vancomycin to VRSA infected mice decreased NO level and MPO activity significantly in liver and kidney (Figure 1-2). 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 (Nikki PYL and Cheng CY, 2008). 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-
Thus, higher level of nitrite by VRSA infection may be due to high production of free radicals. Nanoconjugated vancomycin play the role of antioxidant to prevent the nitrate generation may be through the inhibition of inducible nitric oxide synthase (iNOS) expression (Li W et al. 2006). Hypochlorous acid (HOCl) is generated in the presence of myeloperoxidase and initiates the deactivation of antiproteases and the activation of latent proteases and leads to the cellular damage (Sullivan G et al. 2000). In this study, nanoconjugated vancomycin inhibited the myeloperoxidase activity which was increased due to VRSA infection; suggesting protective role of nanoconjugated vancomycin (Figure 2). These results suggest that either the cellular antioxidants level reached in a higher concentration to exert antioxidant effects or scavenged the free radical produced by the myeloperoxidase (Ogata M et al. 2000). Thus, in addition to the antioxidant system, nanoconjugated vancomycin may indirectly protect liver and kidney from VRSA infection induced oxidative damage. 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 liver and kidney of VRSA infected mice; where as treatment of nanoconjugated vancomycin to VRSA infected mice decreased lipid peroxidation and protein oxidation significantly (Figure 3-4). 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 (Bagchi M et al. 1995). 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, (Reznick AZ and Packer L, 1994) as evidenced by the decreased activity of different antioxidant enzymes like SOD, CAT, GPx, GR, and GST.

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 (Gerster H. 1995). In the present study, the reduced glutathione level was significantly decreased in liver and kidney of VRSA infected mice; where as treatment of nanoconjugated vancomycin to VRSA infected mice increased the GSH level (Figure 5). In this study, it was observed that oxidized glutathione level was increased in liver and kidney of VRSA infected mice, which was ameliorated due to nanoconjugated vancomycin treatment (Figure 6). The decreased GSH levels represent its increased utilization due to 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 due to the increasing production of ROS in form of NO (Sarkar S et al. 1995). In our present study, the increasing levels of GSSG and decreasing GR activity (Figure 10) due to 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$_2^-$) to less dangerous H$_2$O$_2$, which is further degraded by CAT and GPx to water and oxygen (Wetscher G et al. 1995). The results of the present study showed a significant fall in SOD and CAT activities in liver and kidney of VRSA infected group; where as treatment of nanoconjugated vancomycin to VRSA infected mice significantly increased the SOD and CAT activity (Figure 7-8). SOD, dismutate O$_2^-$ and the same in turn is a potent inhibitor of CAT (Ashakumari L and Vijayammal PL, 1996). The depletion in SOD activity was may be due to dispose off the free radicals, produced due to VRSA infection. Beside this, during infection, H$_2$O$_2$ 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 was may be due to inactivation of the enzyme proteins by VRSA infection induced NO generation, depletion of the enzyme substrates, and/or down-regulation of transcription and translation processes.

GPx works non-specifically to scavenge and decompose excess hydroperoxides including H$_2$O$_2$, which may be prevalent under oxidative stress (Somani S. 1996). Glutathione-S-transferase (GST) mainly detoxifies electrophilic compounds (Hemachand T et al. 2002) 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, i.e. GPx, GR, and GST (Figure 9-11) in VRSA infection may be due to increased utilization to scavenge the free radical generation. The results of the present study showed a significant fall of GPx, GR and GST activities in liver and kidney of VRSA infected group; where as treatment of nanoconjugated vancomycin to VRSA infected mice significantly increased the GPx, GR and GST activity in liver and kidney (Figure 9-11). In the present study, it was observed that MDA level (Figure 3) and DNA fragmentation (Figure 12) were significantly elevated in liver and kidney due to VRSA infection. This elevated MDA level decreases GSH level (Figure 5) and SOD activity (Figure 7), which may be associated with DNA fragmentation. In this study, it was observed that DNA fragmentation increased in VRSA infected liver and kidney, which was brought back near to control due to nanoconjugated vancomycin treatment.

5. CONCLUSION

In conclusion, the study described here, liver and kidney is susceptible to \textit{S. aureus} infection through the increased production of nitric oxide which leads to decreased antioxidant status; and nanoconjugated vancomycin protects the liver and kidney from such infection by decreasing free radical generation, lipid and protein damage, and also by increasing the antioxidant status. Hence, the 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.

Abbreviations:
CAT    : Catalase
CFU    : Colony formation unit
CMC    : Carboxymethyl chitosan
CMC-EDBE-FA : Carboxymethyl chitosan-2, 2’ ethylenedioxy bis ethylamine-Folate
CS    : Chitosan
DNA    : Deoxyribonucleic acid
DPA    : Diphenylamine
DTNB    : 5’, 5’-dithio (bis)-2-nitrobenzoic acid
EDTA    : Ethylene diamine tetra acetate
GPx    : Glutathione peroxidase
GR    : Glutathione reductase
GSSG   : Oxidized glutathione
GSH    : Reduced glutathione
GST    : Glutathione-s-transferase
H$_2$O$_2$ : Hydrogen peroxide
MDA    : Malondialdehyde
MPO    : Myeloperoxidase
MRSA    : Methicillin resistant \textit{Staphylococcus aureus}
NADPH   : Nicotinamide adenine dinucleotide phosphate
NO    : Nitric oxide
PC    : Protein carbonyls
ROS    : Reactive oxygen species
SDS    : Sodium dodecyl sulfate

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Approval of interest
Authors declares that there are no conflict of interests.

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