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PROTECTIVE ROLE OF NANOCONJUGATED VANCOMYCIN AGAINST VANCOMYCIN SENSITIVE *STAPHYLOCOCCUS AUREUS* INDUCED OXIDATIVE STRESS AND DNA DAMAGE

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ABSTRACT

Staphylococcus aureus 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 VSSA infection on select makers of oxidative damage and antioxidant status in liver, kidney and spleen. A coagulase positive VSSA strain was used for this study. VSSA infection was developed in Swiss mice by intraperitoneal injection of 5 X 10^6 CFU/ml bacterial solutions. Nanoconjugated vancomycin was treated to VSSA infected mice at a dose of 100 mg/kg b.w/day for 10 days. After decapitation, liver, kidney and spleen were excised from control and experimental groups, homogenized and used for different biochemical estimation. Nitrate level, myeloperoxidse activity, lipid peroxidation, protein oxidation, oxidized glutathione, DNA fragmentation level were increased significantly ($p<0.05$) in liver, kidney and spleen of VSSA 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 VSSA infection induced oxidative stress and DNA damage in liver, kidney and spleen.

INTRODUCTION: *Staphylococcus 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 [1]. More than 90% *S. aureus* strains are resistant to penicillin [2]. Methicillin, a semi synthetic penicillin was used to treat Penicillin Resistant *Staphylococcus aureus* but resistance finally emerge in 1962 [3, 4]. 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 [5]. 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 eights were vancomycin resistant [6]. *S. aureus* causes chronic/relapsing diseases and reported to persist as an opportunistic intracellular organism both *in vitro* and *in vivo* [7]. *S. aureus* were able to survive within phagocytic cells both in polymorphonuclear leukocytes (PMN) and monocytes [8].

Chitosan (CS), the deacetylated form of chitin, is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine linked in a β linkage.[9] CS has been reported to possess immune stimulating properties such as increasing accumulation and activation of macrophages and polymorphonucleus, augmenting antibody responses and inducing production of cytokines [10]. 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 [11]. 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, and complex is called "nanoconjugated vancomycin" and observe its bactericidal activity against *S. aureus*; and reported that CMC-EDBE-FA nanoparticle is non toxic [12, 13].

We also reported that in vivo challenge of VSSA and VRSA for 5 days can produce the highest degree of damage in lymphocyte 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 [14].

Recently, we reported the possible antioxidant effects of nanoconjugated vancomycin against VRSA infection on select makers of oxidative damage, antioxidant status and DNA damage in spleen, liver and kidney [15, 16]. In light of these findings, the present study was focused on the possible protective roles of nanoconjugated vancomycin against VSSA infection on select makers of oxidative damage, antioxidant status, and as well as DNA damage in liver, kidney and spleen.

MATERIALS AND METHODS:

Chemicals and Reagents: Sodium dodecyl sulfate (SDS), 5', 5'-dithio (bis)-2- nitrobenzoic acid (DTNB), standard reduced glutathione (GSH), glutathione reductase (GR), NADPH, Na_4, 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, KH_2PO_4 and K_2HPO_4, alcohol, formaldehyde, paraffin wax, xylene, haematoxylin, eosin, DPX, 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.

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^{o}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.

Bacterial Strain: We used a coagulase positive Vancomycin sensitive *Staphylococcus aureus* (MMC-6) strain that was isolated from human post operative pus sample and was grown at 37°C for overnight in tryptic soy broth [6]. 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 X 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 X 10^6 in 100µl of bacterial suspension [17].

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 [12].

Development of VSSA infection in Swiss mice: VSSA infection was developed in male Swiss mice by intraperitoneal (i.p.) injection of 100µl of bacterial suspension containing 5 X 10^6 CFU/ml according to our previous laboratory report [14].

Experimental Design: VSSA infected mice were treated with nanoconjugated vancomycin for successive 10 days at a dose of 100 mg/kg bw/day. The dose and duration of nanoconjugated vancomycin was selected from our previous laboratory report [14]. The following groups were considered for the experiment:

- Group I: Control,
- Group II: VSSA infection,
- Group III: VSSA infection + 100 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) [18].

Separation and homogenization of Liver, Kidney and Spleen: After decapitation, liver, kidney and spleen 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.

Biochemical Estimation:

- **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 napthyl 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 µ M/mg protein [14].
- **Determination of Myeloperoxidase (MPO) Activity:** 200 µl of sample was reacted with 200 µl substrate (containing H_2O_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 [14]. The MPO activity was expressed in terms of µ M/mg protein.
- **Determination of Lipid Peroxidation (MDA) Level:** Lipid peroxidation of liver, kidney and spleen homogenate was estimated by the method of KarMahapatra *et al*., 2009 [19]. 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.
- **Determination of Protein Carbonyls (PC) Contents:** Protein oxidation was monitored by measuring protein carbonyl contents by derivatization with 2, 4-dinitrophenyl hydrazine (DNPH) [19]. In general, liver, kidney and spleen 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.

- **Determination of Reduced Glutathione (GSH) Level:** Reduced glutathione estimation in liver, kidney and spleen homogenate was performed by the method of KarMahapatra *et al*., 2009 [19]. 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.

- **Determination of Oxidized Glutathione (GSSG) Level:** The oxidized glutathione level in liver, kidney and spleen homogenate was measured after derevatization of GSH with 2-vinylpyidine according to the method of KarMahapatra *et al*., 2009 [19]. In brief, with 0.5 ml sample, 2 µl of 2-vinylpyidine was added and incubates for 1 hr at 37°C. Then the mixture was deprotenized 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.

- **Determination of Super Oxide Dismutase (SOD) Activity:** SOD activity of liver, kidney and spleen homogenate was determined from its ability to inhibit the auto-oxidation of pyrogalol according to KarMahapatra *et al*., 2009 [19]. 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.

- **Determination of Catalase (CAT) Activity:** Catalase activity of liver, kidney and spleen homogenate was measured by the method of KarMahapatra *et al*., 2009 [19]. 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 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 H_2O_2. The level of CAT was expressed as unit/ mg protein.

- **Determination of Glutathione Peroxidase (GPx) Activity:** The GPx activity of liver, kidney and spleen homogenate was measured by the method of KarMahapatra *et al*., 2009 [19]. 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 x 10^3 M^{-1} cm^{-1} at 340 nm. The activity of GPx was expressed in terms of n mol NADPH consumed/min/mg protein.

- **Determination of Glutathione Reductase (GR) Activity:** The GR activity liver, kidney and spleen homogenate was measured by the method of KarMahapatra *et al*., 2009 [19]. 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.

- **Determination of Glutathione-S-Transferase (GST) Activity:** The GST activity of liver, kidney and spleen homogenate was measured by the method of KarMahapatra *et al*., 2009 [19]. 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-dinitrobengene 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.

DNA Fragmentation Assay by diphenylamine (DPA) assay: The diphenylamine (DPA) reaction of liver, kidney and spleen was performed by the method of Paradones *et al.*, 1993 [20]. 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.

Protein estimation: Protein was determined using bovine serum albumin as standard according to Lowry *et al*., 1951 [21].

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.

RESULTS:

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 239.48%, 70.33%, 163.13% and 186.63%, 210.07%, 187.01% in VSSA infected liver, kidney and spleen, respectively, as compared to control group, which were significantly ($P < 0.05$) decreased by 42.48%, 43.87%, 42.15% and 43.84%, 49.65%, 49.92%, respectively, due to treatment of nanoconjugated vancomycin (**Figure 1 & 2**).

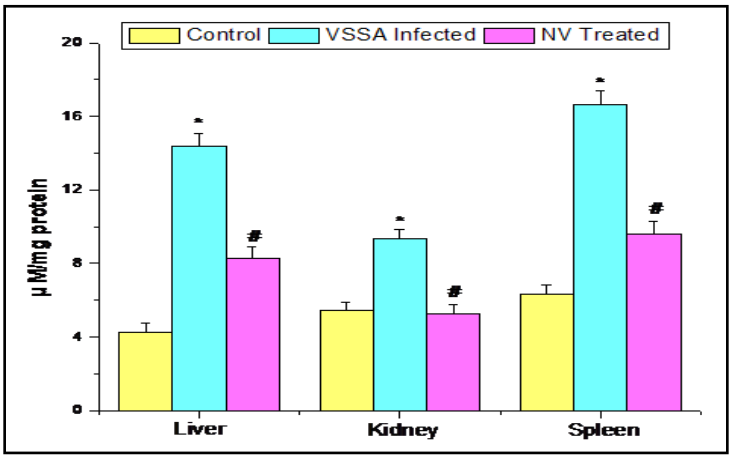

FIGURE 1: NITRATE (NO) GENERATION IN LIVER, KIDNEY AND SPLEEN OF CONTROL, VSSA-INFECTED AND NANOCONJUGATED VANCOMYCIN TREATED GROUP

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 VSSA-infected group

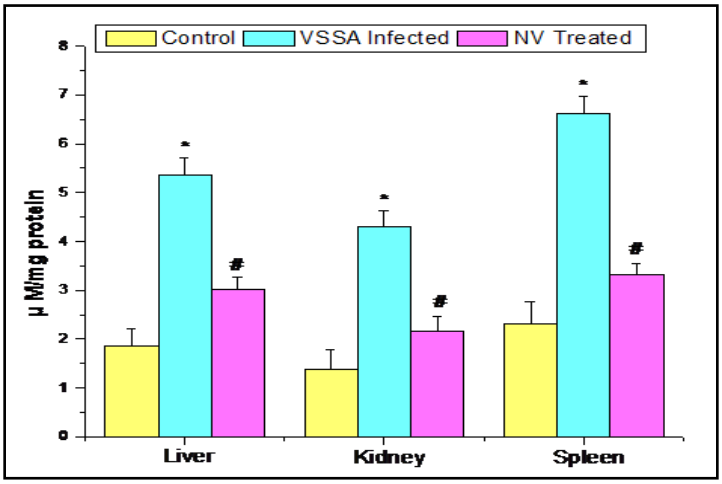

FIGURE 2: MYELOPEROXIDASE (MPO) ACTIVITY IN LIVER, KIDNEY AND SPLEEN OF CONTROL, VSSA-INFECTED AND NANO-CONJUGATED VANCOMYCIN TREATED GROUP

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 VSSA-infected group

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 109.42%, 151.52%, 172.41% and 174.11%, 175.30%, 228.50% in VSSA infected liver, kidney and spleen, respectively, as compared to control group, which were significantly ($P < 0.05$) decreased by 38.85%, 41.13%, 51.02% and 40.76%, 51.33%, 50.20% respectively, due to treatment of nanoconjugated vancomycin (**Figure 3 & 4**).

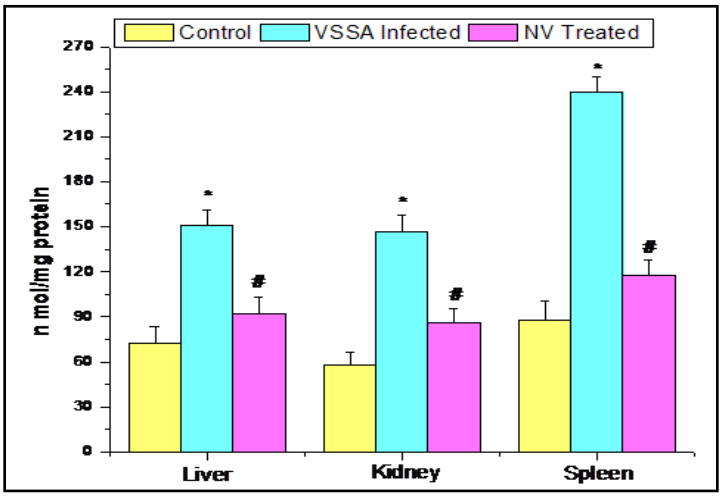

FIGURE 3: LIPID PEROXIDATION (MDA) LEVEL IN LIVER, KIDNEY AND SPLEEN OF CONTROL, VSSA-INFECTED AND NANOCONJUGATED VANCOMYCIN TREATED GROUP

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 VSSA-infected group

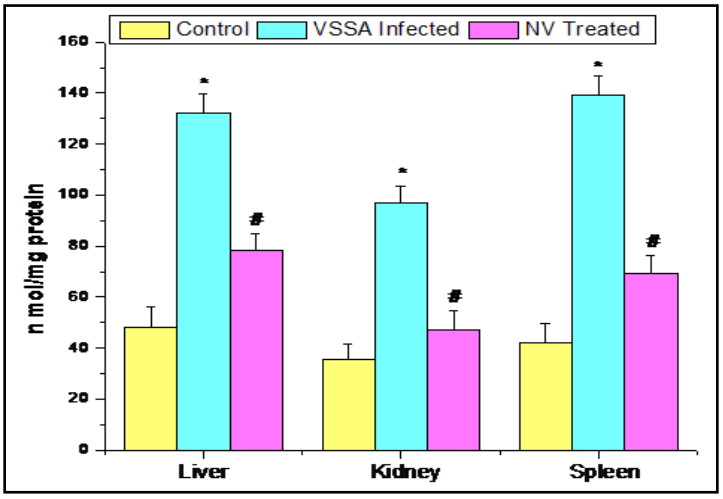

FIGURE 4: PROTEIN CARBONYLS (PC) CONTENTS IN LIVER, KIDNEY AND SPLEEN OF CONTROL, VSSA-INFECTED AND NANOCONJUGATED VANCOMYCIN TREATED GROUP

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 VSSA-infected group

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 44.10%, 42.91% and 49.43% in liver, kidney and spleen of VSSA infected group, respectively, as compared to control; where as the oxidized glutathione level was increases significantly (P <0.05) by 114.16%, 106.15% and 125.01%, respectively, as compared to control.

Treatment of nanoconjugated vancomycin significantly (P <0.05) increased GSH level by 63.01%, 62.10% and 74.17%; and decreased GSSG level significantly (P <0.05) by 45.52%, 45.79% and 46.87%, respectively, in liver, kidney and spleen (**Figure 5 & 6**).

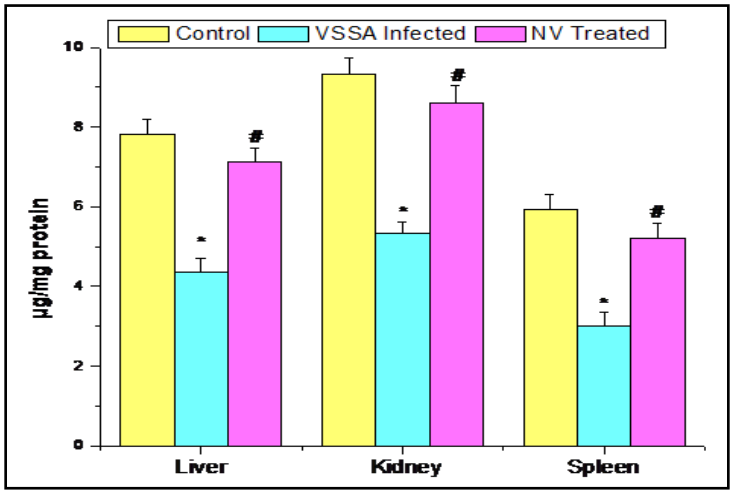

FIGURE 5: REDUCED GLUTATHIONE (GSH) LEVEL IN LIVER, KIDNEY AND SPLEEN OF CONTROL, VSSA-INFECTED AND NANOCONJUGATED VANCOMYCIN TREATED GROUP

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 VSSA-infected group

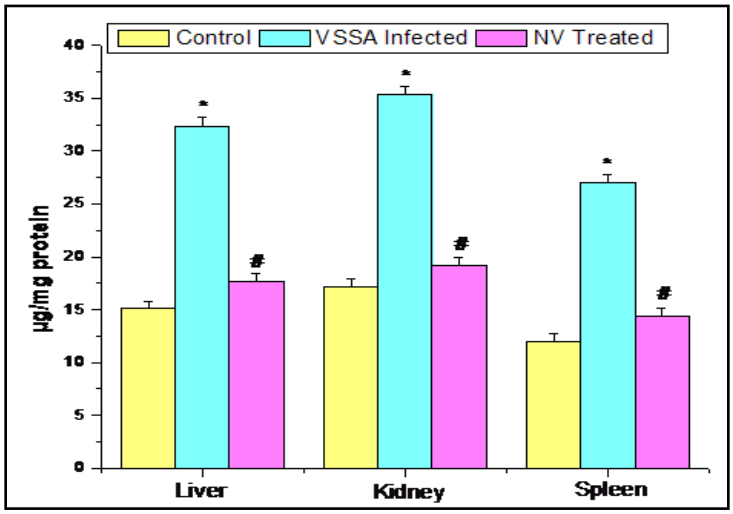

FIGURE 6: OXIDIZED GLUTATHIONE (GSSG) LEVEL IN LIVER, KIDNEY AND SPLEEN OF CONTROL, VSSA-INFECTED AND NANOCONJUGATED VANCOMYCIN TREATED GROUP

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 VSSA-infected group.

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, kidney and spleen of VSSA infected group. SOD and CAT activity were

decreased significantly (P<0.05) by 57.50%, 64.19%, 59.74% and 52.32%, 62.65%, 58.86% in VSSA infected liver, kidney and spleen, respectively, as compared to control group, which were significantly (*P* < 0.05) increased by 100.86%, 161.15%, 127.66% and 64.30%, 127.48%, 110.85%, respectively, due to treatment of nanoconjugated vancomycin (**Figure 7 & 8**).

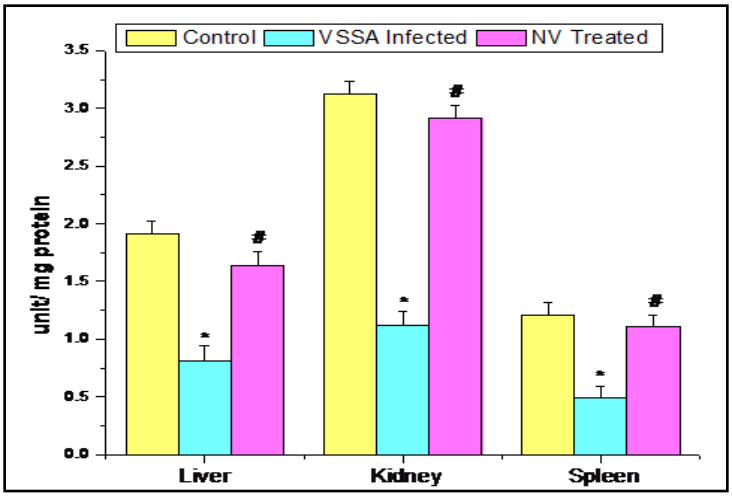

FIGURE 7: SUPEROXIDE DISMUTASE (SOD) ACTIVITY IN LIVER, KIDNEY AND SPLEEN OF CONTROL, VSSA-INFECTED AND NANOCONJUGATED VANCOMYCIN TREATED GROUP

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 VSSA-infected group

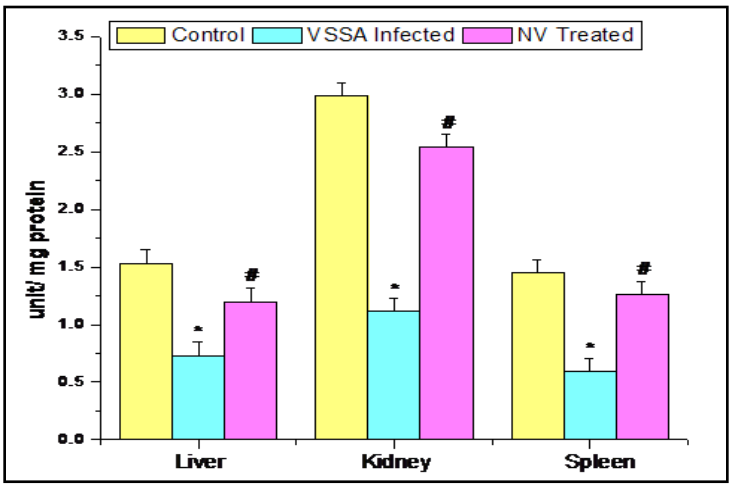

FIGURE 8: CATALASE (CAT) ACTIVITY IN LIVER, KIDNEY AND SPLEEN OF CONTROL, VSSA-INFECTED AND NANOCONJUGATED VANCOMYCIN TREATED GROUP

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 VSSA-infected group

Glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-s-transferase activity: Glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-s-transferase (GST) were measured to understand the antioxidant enzymes status in spleen of VSSA infected group. GPx, GR and GST activity were decreased significantly (P<0.05) by 46.34%, 74.41%, 52.23%; 51.74%, 55.54%, 53.07% and 53.11%, 73.25%, 41.98% in VSSA infected liver, kidney and spleen, respectively, as compared to control group, which were significantly (*P* < 0.05) increased by 60.05%, 240.33%, 73.78%; 86.90%, 95.99%, 84.57% and 84.94%, 216.09%, 53.74%, respectively, due to treatment of nanoconjugated vancomycin (**Figure 9, 10 & 11**).

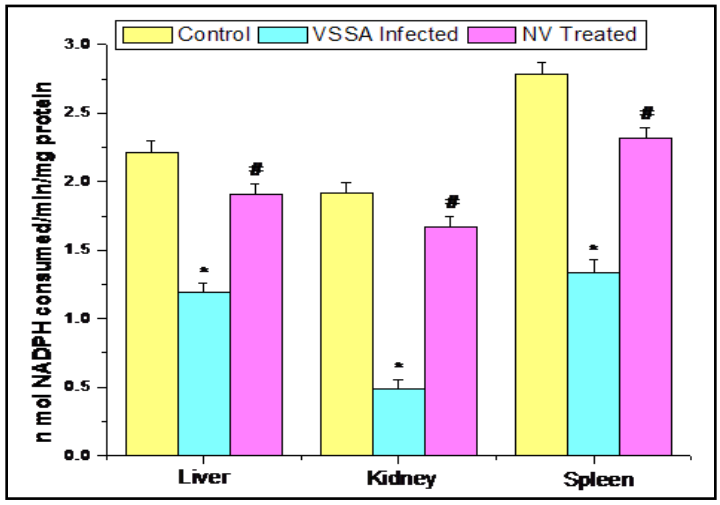

FIGURE 9: GLUTATHIONE PEROXIDASE (GPX) ACTIVITY IN LIVER, KIDNEY AND SPLEEN OF CONTROL, VSSA-INFECTED AND NANOCONJUGATED VANCOMYCIN TREATED GROUP

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 VSSA-infected group.

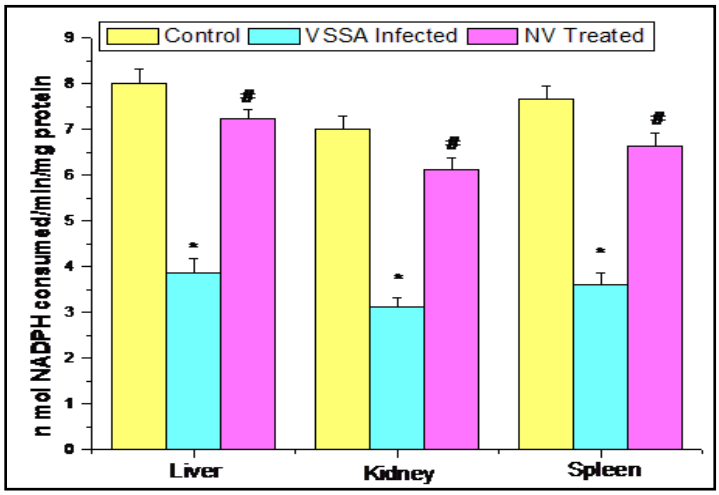

FIGURE 10: GLUTATHIONE REDUCTASE (GR) ACTIVITY IN LIVER, KIDNEY AND SPLEEN OF CONTROL, VSSA-INFECTED AND NANOCONJUGATED VANCOMYCIN TREATED GROUP

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 VSSA-infected group

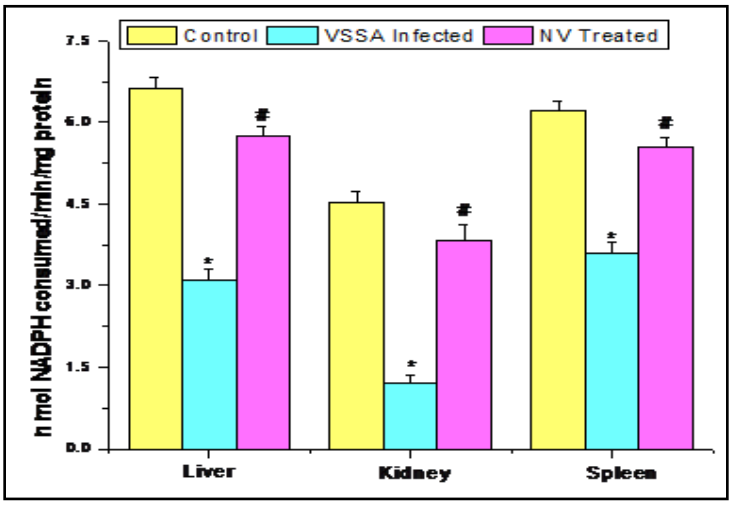

FIGURE 11: GLUTATHIONE-S-TRANSFERASE (GST) ACTIVITY IN LIVER, KIDNEY AND SPLEEN OF CONTROL, VSSA-INFECTED AND NANOCONJUGATED VANCOMYCIN TREATED GROUP

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 VSSA-infected group

DNA fragmentation: DNA fragmentation is an indicator of apoptotic cell death; hence the quantitative DNA fragmentation in liver, kidney and spleen was evaluated in this study. Quantitative DNA fragmentation in all groups of liver, kidney and spleen was evaluated by diphenylamine (DPA) assay spectrophotometrically. VSSA infection produced 695.99%, 921.05% and 448.44% fragmented DNA, where as control group showed negligible fragmented DNA in liver, kidney and spleen that was associated significantly ($P < 0.05$). Treatment of nanoconjugated vancomycin significantly ($P < 0.05$) decreased DNA fragmentation by 68.10%, 75.38% and 61.23%, respectively (**Figure 12**).

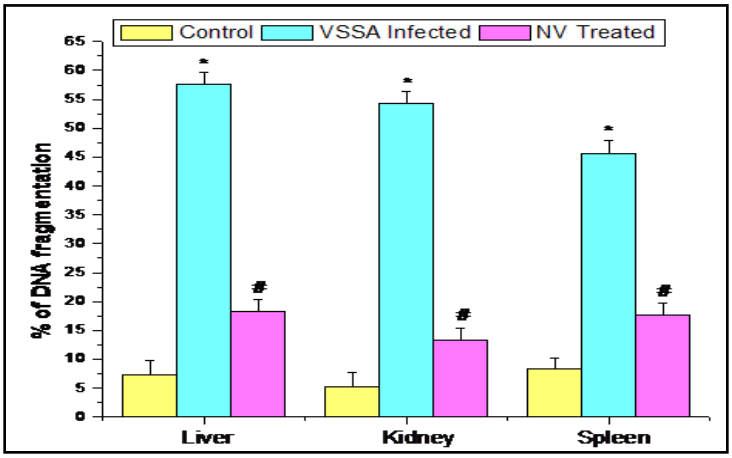

FIGURE 12: QUANTITATIVE ESTIMATION OF DNA FRAGMENTATION ASSAY BY DIPHENYLAMINE (DPA) ASSAY IN LIVER, KIDNEY AND SPLEEN OF CONTROL, VSSA-INFECTED, AND NANOCONJUGATED VANCOMYCIN TREATED GROUP

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 VSSA-infected group.

DISCUSSION: The result of our study demonstrate that, VSSA 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, kidney and spleen which were ameliorated by treatment of nanoconjugated vancomycin (Figure 1-11). More over DNA damage assessed by DPA assay due to VSSA infection was also observed in liver, kidney and spleen, which were protected by treatment of nanoconjugated vancomycin (Figure 12).

In this study, significant elevation of nitrate level and MPO activity in liver, kidney and spleen was observed in VSSA infected mice; which were decreased in nanoconjugated vancomycin treated group. Treatment of nanoconjugated vancomycin to VSSA infected mice decreased NO level and MPO activity significantly in spleen (Figure 1 & 2). Nitric oxide (NO) is a free radical synthesized by nitric oxide synthase (NOS). NOS are composed of two identical monomers with molecular weights ranging from 130 to 160 kDa [22]. 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 [14].

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 [23]. 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 [24].

In this study, nanoconjugated vancomycin inhibited the myeloperoxidase activity which was increased due to VSSA 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 [25]. Thus, in addition to the antioxidant system, nanoconjugated vancomycin may indirectly protect liver, kidney and spleen from VSSA 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, kidney and spleen of VSSA infected mice; whereas treatment of nanoconjugated vancomycin to VSSA 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 [26].

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 [27], 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 [28]. In the present study, the reduced glutathione level was significantly decreased in liver, kidney and spleen of VSSA infected mice; whereas treatment of nanoconjugated vancomycin to VSSA 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 VSSA infected mice, which was ameliorated due to nanoconjugated vancomycin treatment (Figure 6). The decreased GSH levels represent its increased utilization due to VSSA 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 [29]. In our present study, the increasing levels of GSSG and decreasing GR activity (Figure 10) due to VSSA 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 ($O_2^{\cdot -}$) to less dangerous H_2O_2, which is further degraded by CAT and GPx to water and oxygen [30]. The results of the present study showed a significant fall in SOD and CAT activities in liver, kidney and spleen of VSSA infected group; whereas treatment of nanoconjugated vancomycin to VSSA infected mice significantly increased the SOD and CAT activity (Figure 7 & 8). SOD, dismutate $O_2^{\cdot -}$ and the same in turn is a potent inhibitor of CAT [31].

The depletion in SOD activity was 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 was may be due to inactivation of the enzyme proteins by VSSA 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_2O_2, which may be prevalent under oxidative stress [32]. Glutathione-S-transferase (GST) mainly detoxifies electrophilic compounds and has a well-established role in protecting cells from mutagens and carcinogens as a free radical scavenger along with glutathione [33].

In the present study, the significant decreasing of GSH level and GSH-dependent enzymes, i.e. GPx, GR, and GST (Figure 9, 10 & 11) in VSSA 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,

kidney and spleen of VSSA infected group; whereas treatment of nanoconjugated vancomycin to VSSA infected mice significantly increased the GPx, GR and GST activity in liver, kidney and spleen (Figure 9, 10 & 11). In the present study, it was observed that MDA level (Figure 3) and DNA fragmentation (Figure 12) were significantly elevated in liver, kidney and spleen due to VSSA 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 VSSA infected liver, kidney and spleen, which was brought back near to control due to nanoconjugated vancomycin treatment.

CONCLUSION: In conclusion, the study described here, liver, kidney and spleen is susceptible to *S. aureus* infection through the increased production of nitric oxide which leads to decreased antioxidant status; and nanoconjugated vancomycin protects the liver, kidney and spleen 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.

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