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In vitro Staphylococcus aureus-induced oxidative stress in mice murine peritoneal macrophages: a duration-dependent approach

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PEER REVIEW

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Comments

This work is interesting and contains novel data. This work can be future referenced in the field of microbiology and tropical medicine. Its eminent finding is in the clinical chemistry finding on the antioxidant assessment and corresponding bacterial pathogen.
Details on Page S304

ABSTRACT

Objective: To evaluate the free radical generation and status of the antioxidant enzymes in murine peritoneal macrophage during *in vitro* vancomycin sensitive *Staphylococcus aureus* (VSSA) treatment with different time interval.
Methods: Peritoneal macrophages were treated with 5×10^6 CFU/mL VSSA cell suspension *in vitro* for different time interval (1, 2, 3, 6, 12, and 24 h) and superoxide anion generation, NADPH oxidase activity, myeloperoxidase activity, nitric oxide generation, antioxidant enzyme status and components of glutathione cycle were analyzed.
Results: Superoxide anion generation, NADPH oxidase activity, myeloperoxidase activity and nitric oxide generation got peak at 3 h, indicating maximum free radical generation through activation of NADPH oxidase in murine peritoneal macrophages during VSSA infection. Reduced glutathione level, glutathione peroxidase, glutathione reductase, and glutathione-s-transferase activity were decreased significantly ($P<0.05$) with increasing time of VSSA infection. But the oxidized glutathione level was time dependently increased significantly ($P<0.05$) in murine peritoneal macrophages. All the changes in peritoneal macrophages after 3 h *in vitro* VSSA treatment had no significant difference.
Conclusions: From this study, it may be summarized that *in vitro* VSSA infection not only generates excess free radical but also affects the antioxidant status and glutathione cycle in murine peritoneal macrophages.

KEYWORDS

Antioxidant enzyme, Oxidative stress, Peritoneal macrophage, Vancomycin sensitive *Staphylococcus aureus*

1. Introduction

Staphylococcus aureus (*S. aureus*) often causes chronic or relapsing diseases. It was reported to persist as an opportunistic intracellular organism both *in vitro* and *in vivo*. *S. aureus* were able to survive within phagocytic cells both in polymorphonuclear leukocytes and monocytes. To induce an infection in host the pathogenic must cope with their changing environment and have to attack continuously the host to weak the immune system. Recent reviews discussed about the molecular mechanism of the survival of bacteria in host phagocytic cell. The generation of reactive oxygen species (ROS) by phagocytic cells is thought to be an important component of the host's immunity against bacterial infection[1]. Generation of ROS and reactive oxygen intermediates are thought to be part of the oxygen dependent bactericidal mechanisms in the phagocytic cell. After engulfment of bacteria by phagocytosis, the generation of ROS occurs during oxidative stress resulting imbalance between ROS and antioxidant level results in killing of the phagocytosed microbe. Anti-oxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) form part of a defense mechanism helps to protect bacteria from oxygen toxicity[2].

Macrophages are ubiquitous mononuclear phagocytes in mammalian tissues. The peritoneal macrophages are

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representative of other macrophage populations, and also easily available in mice in greater amounts than blood phagocytes[3]. The immune cell functions are specially linked to ROS generation and are strongly influenced by the redox potential. Therefore the oxidant/antioxidant balance is an important determinant of immune cell activity. The antioxidant levels in immune cells play a pivotal role in protecting them against oxidative stress and therefore preserving their adequate function. In our previous laboratory report, it was clearly established that *in vivo* challenge of vancomycin sensitive and resistant *S. aureus* for 5 d can produce the highest degree of damage in lymphocyte through the increased production of nitric oxide (NO) and TNF-α that leads to decreased antioxidant status in cell[4]. Therefore, the present study was performed to focus on the time-dependent vancomycin sensitive *S. aureus* (VSSA) infection-induced toxicity in murine peritoneal macrophages.

2. Materials and methods

2.1. Chemicals and reagents

Phorbol myristate acetate (PMA), quercetin, gallic acid, horse heart cytochrome-*c*, sodium dodecyl sulfate (SDS), 2,4-dinitrophenyl hydrazine (DNPH), 5′, 5′-dithio (bis)-2-nitrobenzoic acid (DTNB), standard reduced glutathione (GSH), glutathione reductase (GR), NADPH, NADPH Na_4, oxidized glutathione (GSSG) were obtained from Sigma, USA. RPMI 1640, fetal bovine serum (FBS), heparin, ethylene diamine tetra acetate (EDTA), sodium chloride (Nacl), SDS, sucrose, magnesium chloride ($MgCl_2$), sodium azide (NaN_3), tryptic soy broth were purchased from Himedia, India. Tris-HCl, Tris buffer, potassium dihydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4), sodium hydroxide (NaOH), sodium acetate, ammonium acetate, alcohol, sulfanilamide, phosphoric acid, N-C-1 napthyl ethylene diamine dihydrochloride, glycerol, boric acid, 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. Bacterial strain

A pathogenic VSSA (MMC-6) was selected for this study. This strain was isolated from post operative pus sample and clinically identified in our laboratory[5].

2.3. Isolation of the peritoneal macrophages and cell culture

Experiments were performed using Swiss male mice 6-8 weeks old, weighing 20-25 g. The animals were fed with standard pellet diet and water were given *ad libitum*, and the mice were 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 the experiment was approved by the ethical committee of Vidyasagar University. All efforts were made to minimize animal suffering and to reduce the number of animals used. Macrophages were isolated by peritoneal lavage from male Swiss mice, after 24 h injection of 2 mL of 4% starch according to our previous lab report[6]. In brief, the peritoneal cavity were washed with ice cold phosphate buffer saline (PBS) supplemented with 20 IU/mL heparin and 1 mmol/L EDTA. Care was taken not to cause internal bleeding while collecting macrophages in the exudates. The cells were then cultured in 60 mm Petri dishes in RPMI-1640 media supplemented with 10% FBS, 50 μg/mL gentamycin, 50 μg/mL penicillin and 50 μg/mL streptomycin for 24 h at 37 °C in a humidified atmosphere of 95% air/5% CO_2 atmosphere in CO_2 incubator. Non-adherent cells were removed by vigorously washing three times with ice-cold PBS. Differential counts of the adherent cells used for the experiments were determined microscopically after staining with Giemsa and the cell viability evaluated by trypan blue exclusion was never below 95%[6,7].

2.4. Preparation of bacterial suspension

This bacterial strain was grown at 37 °C overnight in tryptic soy broth. The bacterial culture was centrifuged at 15000 r/min for 15 min. The pallet was resuspended and washed with sterile PBS. Using a UV-spectrophotometer (Schimadzu, USA) at an absorbance of 620 nm, the viable bacterial count was adjusted to approximately 10^9 CFU/mL, which corresponded to an optical density of 1.6. The bacterial suspension was adjusted by serial dilution in PBS to final concentration of approximately 5×10^6 in 100 μL of bacterial suspension[8].

2.5. Experimental design and sample preparation

The peritoneal macrophages were divided into 6 groups for six different duration dependent experiments as follows: Group I: 1 h; Group II: 2 h; Group III: 3 h; Group IV: 6 h; Group V: 12 h and Group VI: 24 h. Each group is divided into two sub-groups, one is control and another is for 5×10^6 CFU/mL VSSA treatment. Each sub-group contained 6 Petri dishes (4×10^6 cells in each). The cells of each Petri dishes were maintained in RPMI 1640 media supplemented with 10% FBS, 50 μg/mL gentamycin, 50 μg/mL penicillin and 50 μg/mL streptomycin at 37 °C in a 95% air/5% CO_2 atmosphere in CO_2 incubator. The concentration of VSSA was selected according to Ives *et al*[9]. After the treatment schedule the cells were collected from the Petri dishes separately and centrifuged at 2200 r/min for 10 min at 4 °C. Then the supernatant was collected in separate micro centrifuge tube and the cells were washed twice with 50 mmol/L PBS (pH 7.4). The pallets were lysed with hypotonic lysis buffer (10 mmol/L tris, 1 mmol/L EDTA and Titron X-100, pH 8.0) for 45 min at 37 °C and then processed for the biochemical estimation[10]. Intact cells were used for superoxide anion generation and NADPH oxidase activity.

2.6. Biochemical estimation

2.6.1. Assessment of superoxide anion generation

Superoxide anion generation was determined by a standard assay[6]. Briefly, 0.1 μg/mL of PMA (Sigma), a potent macrophage stimulant, and 0.12 mmol/L horse heart cytochrome-*c* (Sigma) were added to isolated cell suspensions after treatment schedule, and washed with PBS. Cytochrome-*c* reduction by generated superoxide was then determined by spectrophotometric absorbance at a 550 nm wavelength. Results were expressed as nmol of cytochrome-*c* reduced/min, using extinction coefficient 2.1×10^{4} M^{-1} cm^{-1}.

2.6.2. NADPH oxidase activity

After the treatment schedule, the macrophages of different groups were prewarmed in Krebs ringer buffer with 10 mmol/L glucose at 37 °C for 3 min. PMA (0.1 μg/ mL) prewarmed at 37 °C for 5 min was added, and the reaction was stopped by putting in ice. Centrifugation was carried out at 1 500 r/min for 5 min and the resultant pellet was resuspended in 0.34 mol/L sucrose. The cells were then lysed with hypotonic lysis buffer. Centrifugation was carried out at 3 000 r/min for 10 min and the supernatant used to determine enzyme activity. NADPH oxidase activity was determined spectrophotometrically by measuring cytochrome-*c* reduction at 550 nm. The reaction mixture contained 10 mmol/L phosphate buffer (pH 7.2), 100 mmol/L NaCl, 1 mmol/L $MgCl_2$, 80 μmol/L cytochrome-*c*, 2 mmol/L NaN_3 and 100 μL of supernatant (final volume 1 mL). A suitable amount of NADPH (10-20 μL) was added last to initiate the reaction[11].

2.6.3. NO generation

NO generation in cell lysate was assessed according to our previous laboratory report[12]. Sodium nitroprusside (100 mmol/L) 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 napthyl 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 μmol/mg protein.

2.6.4. Myeloperoxidase (MPO) activity

A total of 200 μL of cell lysate 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 of 1 mol/L sulfuric acid and reading was taken at 492 nm in a spectrophotometer. The MPO activity was expressed in terms of μmol/L/mg protein[13].

2.6.5. Determination of lipid peroxidation

Lipid peroxidation was estimated in cell lysate according to our previous laboratory report[14]. Briefly, the reaction mixture contained tris-HCl buffer (50 mmol/L, pH 7.4), tert-butyl hydroperoxide (500 μmol/L in ethanol) and 1 mmol/L $FeSO_4$. After incubating the samples at 37 °C for 90 min, the reaction was stopped by adding 0.2 mL of 8% 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% thiobutiric acid and further heating the mixture at 95 °C for 45 min. After cooling, samples were centrifuged, and the thiobutiric acid reactive substances were measured in supernatants at 532 nm by using 1.53×10^{5} M^{-1} cm^{-1} as extinction coefficient. The levels of lipid peroxidation were expressed in terms of μmol/mg protein.

2.6.6 Protein carbonyls (PC) contents

Protein oxidation was monitored by measuring PC contents by derivatization with DNPH[15]. In general, cell lysate proteins in 50 mmol/L potassium phosphate buffer, pH 7.4, were derivatized with DNPH (21% in 2 mol/L HCl). Blank samples were mixed with 2 mol/L HCl incubated for 1 h in the dark; protein was precipitated with 20% trichloro acetic acid. Underivatized proteins were washed with an ethanol:ethyl acetate mixture (1:1). Final pellets of protein were dissolved in 6 mol/L guanidine hydrochloride and absorbance was measured at 370 nm. PC content was expressed in terms of μmol/mg protein.

2.6.7. Determination of GSH level

GSH estimation in the cell lysate was performed according to our previous laboratory report[14]. The required amount of the cell lysate was mixed with 25% of trichloroacetic acid and centrifuged at 7 500 r/min for 15 min to settle the precipitated proteins. The supernatant was aspirated and diluted to 1 mL with 0.2 mol/L sodium phosphate buffer (pH 8.0). Later, 2 mL of 0.6 mmol/L 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 GSH. The levels of GSH were expressed as μg of GSH/mg protein.

2.6.8. Determination of GSSG level

The GSSG level was measured after derevatization of GSH with 2-vinylpyidine according to our previous laboratory report[14]. In brief, with 0.5 mL cell lysate, 2 μL 2-vinylpyidine was added and incubates for 1 h at 37 °C. Then the mixture was deprotenized with 4% sulfosalicylic acid and centrifuged at 3750 r/min 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.

2.6.9. Activity of SOD

SOD activity was determined from its ability to inhibit the auto-oxidation of pyrogalol according to our previous laboratory report[14]. The reaction mixture contained 50 mmol/L tris (hydroxymethyl) amino methane (pH 8.2), 1

mmol/L diethylenetriamine penta acetic acid, and 20-50 μL of cell lysate. The reaction was initiated by addition of 0.2 mmol/L pyrogalol, and the absorbance measured kinetically at 420 nm at 25 °C for 3 min. SOD activity was expressed as IU/mg protein.

2.6.10. Activity of CAT

CAT activity was measured in the cell lysate according to our previous laboratory report[14]. The final reaction volume of 3 mL contained 0.05 mol/L tris-buffer, 5 mmol/L EDTA (pH 7.0), and 10 mmol/L H_2O_2 (in 0.1 mol/L potassium phosphate buffer, pH 7.0). About 50 μL aliquot of the cell lysates were added to the above mixture. The rate of change of absorbance per min at 240 nm was recorded. CAT 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 in terms of μmol H_2O_2 consumed/(min · mg) protein.

2.6.11. Activity of glutathione peroxidase (GPx)

The GPx activity was measured according to our previous laboratory report[14]. The reaction mixture contained 50 mmol/L potassium phosphate buffer (pH 7.0), 1 mmol/L EDTA, 1 mmol/L sodium azide, 0.2 mmol/L NADPH, 1 IU GR and 1 mmol/L GSH. 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 mmol/L H_2O_2. Absorbance at 340 nm was recorded for 5 min. Values were expressed as nmol of NADPH oxidized to NADP by using the extinction coefficient of 6.2×10^3 M^{-1} cm^{-1} at 340 nm. The activity of GPx was expressed in terms of nmol NADPH consumed/(min · mg) protein.

2.6.12. Activity of GR

The GR activity was measured according to our previous laboratory report[14]. The tubes for enzyme assay were incubated at 37 °C and contained 2.0 mL of 9 mmol/L GSSG, 0.02 mL of 12 mmol/L NADPH, Na_4, 2.68 mL of 1/15 mol/L 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 nmol NADPH consumed/(min · mg) protein.

2.6.13. Activity of glutathione-s-transferase (GST)

The activity of GST was measured according to our previous laboratory report[14]. The tubes of enzyme assay were incubated at 25 °C and contained 2.85 mL of 0.1 mol/L potassium phosphate (pH 6.5) containing 1 mmol/L of GSH, 0.05 mL of 60 mmol/L 1-chloro-2, 4-dinitrobengene 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 nmol NADPH consumed/(min · mg) protein.

2.6.14. Protein estimation

Protein was determined using bovine serum albumin as standard according to our previous laboratory report[16].

2.7. Statistical analysis

The data were expressed as mean±SEM, n=6. Comparisons between the means of control and VSSA treated group of each time interval were made by One-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with student's t-tests, $P<0.05$ as a limit of significance.

3. Results

3.1. Superoxide radical generation

In vitro time dependent superoxide anion generation due to VSSA treatment in peritoneal macrophages is presented in Table 1. Significant increases ($P<0.05$) in superoxide anion generation, as indicated by reduction of cytochrome-c, were observed compared to control levels at all respective time interval. Highest amount of radical generation was found at 3 h treatment schedule and it was 148.17% high than control group in this time period. After that, it was gradually decreased up to 65.12% than control group at 24 h.

3.2. NADPH oxidase activity

NADPH oxidase activation is the key factor to generate the superoxide anion in cell. NADPH oxidase activity was also time dependently increased significantly ($P<0.05$) from 1 h to 24 h VSSA treatment in macrophage with comparison to their respective control group and highest level of activity of this enzyme was noted at 3 h. After that the activation was slightly decreased (Table 1).

3.3. Nitrate generation level

Nitrite generation occurred by inducible nitrite synthase can then combine with superoxide and be able to generate a product which have much more toxicity such as peroxinitrite ($ONOO^-$). NO generation in macrophage was time dependently increased significantly ($P<0.05$) from 1 h to 24 h VSSA treatment in macrophage comparison to their respective control group and highest level of activity of this enzyme was noted at 3 h. After that NO generation was slightly decreased (Table 1).

3.4. MPO activity

MPO is an important enzyme to produce hypochlorus acid in cellular system that leads to oxidative damage. So, it is an important determinant to establish the free radical generation in peritoneal macrophage. MPO activity in VSSA treated murine peritoneal macrophages was time dependently increased significantly ($P<0.05$) from 1 h to 24 h VSSA treatment in comparison to their respective control group and highest level of activity of this enzyme was noted at 3 h. After that the activation was slightly decreased (Table 1).

3.5. Lipid peroxidation level

Lipid peroxidation in terms of MDA is an important

Table 1
Values of different biochemical parameters of control and VSSA treated group.

Parameters	1h		2h		3h		6h		12h		24h	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Superoxide anion generation [(nmol/(min · 10^6 cells)]	46.871±1.057	70.745±0.997	48.698±1.167	92.458±0.942*	47.872±0.985	128.437±0.853*	50.412±1.48	117.399±0.796*	47.345±0.956	93.991±0.979*	45.315±1.118	79.265±0.859*
NADPH oxidase activity [(nmol/(min · 10^5 cells)]	46.871±1.057	68.745±0.997*	48.698±1.167	88.458±0.942*	47.872±0.985	108.437±0.853*	50.412±1.48	97.399±0.796*	47.345±0.956	93.991±0.979*	45.315±1.118	83.265±0.859*
NO generation (μmol/mg protein)	23.13±1.975	40.953±1.605*	24.302±1.663	59.443±2.910*	23.769±1.662	117.045±2.186*	25.493±1.261	96.567±1.246*	22.663±1.115	82.3175±1.451*	23.667±0.989	69.301±0.972*
MPO activity (μmol/mg protein)	17.13±1.975	35.953±1.605*	17.302±1.663	49.443±2.910*	17.769±1.662	67.045±2.186*	17.493±1.261	61.567±1.246*	17.663±1.115	55.317±1.451*	17.667±0.989	49.301±0.972*
MDA level (μmol/mg protein)	27.13±1.975	35.953±1.605*	27.302±1.663	49.443±2.910*	27.769±1.662	69.045±2.186*	27.493±1.261	60.567±1.246*	27.663±1.115	54.375±1.451*	27.667±0.989	49.301±0.972*
PC level (μmol/mg protein)	17.13±1.975	31.953±1.605*	17.302±1.663	39.443±1.910*	17.769±1.662	44.567±1.186*	17.493±1.261	42.375±1.246*	17.663±1.115	40.301±1.451*	17.667±0.989	38.012±0.972*
GSH level (μg of GSH/mg protein)	1.415±0.045	0.885±0.055*	1.463±0.018	0.677±0.023*	1.385±0.045	0.308±0.029*	1.395±0.035	0.455±0.065*	1.352±0.035	0.595±0.065*	1.401±0.027	0.701±0.053*
GSSG level (μg of GSSG/mg protein)	0.685±0.055	1.015±0.055*	0.677±0.018	1.063±0.023*	0.678±0.045	1.185±0.029*	0.675±0.035	1.095±0.065*	0.675±0.035	0.992±0.065*	0.671±0.027	0.941±0.053*
SOD activity (IU/mg protein)	33.679±0.955	29.664±0.891	33.014±0.865	17.105±0.862*	32.541±0.925	12.569±0.957*	33.472±0.954	14.593±0.903*	31.052±0.942	17.362±0.875*	30.256±0.842	19.321±0.972*
CAT activity (μmol H_2O_2 consumed/(min · mg) protein)	16.325±0.286	13.256±0.312	16.236±0.214	10.062±0.232*	16.324±0.274	8.012±0.189*	16.239±0.189	8.735±0.261*	16.148±0.342	9.365±0.232*	16.023±0.232	11.002±0.341*
GPx activity (nmol NADPH consumed/min · mg protein)	6.325±0.086	4.256±0.092*	6.236±0.084	4.062±0.092*	6.324±0.074	3.212±0.089*	6.239±0.089	3.335±0.061*	6.148±0.082	3.765±0.092*	6.023±0.082	4.002±0.091*
GR activity (nmol NADPH consumed/min · mg protein)	9.325±0.216	7.256±0.312*	9.236±0.214	6.062±0.232*	9.324±0.214	4.612±0.189*	9.239±0.189	4.835±0.261*	9.148±0.242	5.765±0.232*	9.023±0.232	6.002±0.341*
GST activity (nmol NADPH consumed/min · mg protein)	12.325±0.286	7.256±0.312*	12.236±0.214	6.062±0.232*	2.324±0.274	3.612±0.189*	12.239±0.189	4.835±0.261*	12.148±0.342	6.765±0.232*	12.023±0.232	7.002±0.341*

Values are expressed as mean±SEM, n=6. *Significant difference (P<0.05) in VSSA treated group compared to control group at respective time interval.

determination of cellular injury. MDA level in macrophages of each group are shown in Table 1. MDA levels were increased significantly (P<0.05) in time dependent manner in VSSA treated macrophage compared to respective control. Starting with 31.31% increase of MDA level at 1 h in macrophage, it gone up to 123.11% at 24 h but highest degree of damage was found at 3 h (171.17%) relative to their respective control group.

3.6. Protein oxidation level

Lipid peroxidation in terms of PC is an important determination of cellular injury. PC level in macrophages of each group are shown in Table 1. PC levels were increased significantly (P<0.05) in time dependent manner in VSSA treated macrophage compared to respective control. Starting with 37.31% increase of PC level at 1 h in macrophage, it gone up to 139.19% at 24 h but highest degree of damage was found at 3 h (161.44%) relative to their respective control group.

3.7. GSH level

The GSH levels were decreased significantly (P<0.05) at all duration of VSSA treatment; 35.35%, 51.22%, 75.36%, 65.37%, 53.99%, and 47.91%. GSH levels were decreased due to *in vitro* VSSA treatment for 1 h, 2 h, 3 h, 6 h, 12 h and 24 h duration, respectively, in peritoneal macrophages compared with respective control cells (Table 1). Maximum decreased GSH level was found at 3 h VSSA treatment.

3.8. GSSG level

The GSSG levels were increased significantly (P<0.05) at all duration of VSSA treatment: 42.43%, 51.57%, 76.52%, 62.78%, 43.69%, and 37.20%. GSSG levels were increased due to *in vitro* VSSA treatment for 1 h, 2 h, 3 h, 6 h, 12 h and 24 h duration, respectively, in peritoneal macrophages compared with respective control cells (Table 1). Maximum decreased GSH level was found at 3 h VSSA treatment.

3.9. SOD activity

SOD catalyzes the breakdown of superoxide radical into oxygen and H_2O_2. SOD activity was decreased significantly (P<0.05) from 2 h to 24 h of the study in macrophages compared to their respective control group, which is presented in Table 1. The maximum decreased level of SOD was noted at 3 h and it was 57.31% lower than the control group in this time dependent study. After that the SOD level increases slightly and the decrease was 42.17% compared to control group at 12 h.

3.10. CAT activity

CAT catalyzes the conversion of H_2O_2 to oxygen and water. CAT activity was decreased significantly (P<0.05) from 2 h to 24 h of the time dependent study compared with their respective control group, which is presented in Table 1. The maximum decreased level of CAT was noted at 3 h and it was 48.89% lower than the control group in the time dependent study. After that CAT level increased slightly at 12 h and it was 38.01% lower than the control group.

3.11. GPx activity

GPx activity was decreased significantly (P<0.05) at all duration of the VSSA treatment (Table 1). GPx activity decreased maximum (39.12% decreased activity than control) at 3 h.

3.12. GR activity

GR activity was decreased significantly (P<0.05) at all duration of the VSSA treatment (Table 1). GR activity was decreased maximum at 3 h and at this time it was 45.72% lower than control group.

3.13. GST activity

GST activity was found decreased significantly (P<0.05) starting from 1 h (21.39% lower than control group) and upto 24 h (89.71% lower than control group) *in vitro* VSSA treatment; but lowest level of activity of this enzyme was noted at 3 h (105.47% lower than control group) (Table 1).

4. Discussion

Cells are provided with very strong biological antioxidant

defense mechanisms by nature. These include a variety of enzymatic and non-enzymatic 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[17].

In the present study, *in vitro* VSSA treatment can generate excess amount of superoxide anion through excess activation of NADPH oxidase in time dependent manner. The activated NADPH oxidase transports electrons from NADPH on the cytoplasmic side of the membrane to oxygen in the extra cellular fluid to form superoxide anion[18]. This excess superoxide anion leads to oxidative damage of macromolecules, lipid, and lowers antioxidant system in cell. As a result, increased lipid peroxidation (MDA level) and protein oxidation was found in time dependent manner at all duration of the *in vitro* VSSA treatment. Glutathione system is an important antioxidant system in cell and involved in protection against free radicals, peroxides, and toxic compounds and their reactive intermediates formed intracellularly either spontaneously or enzymatically in cellular systems. The enzymatic conjugation of these toxic substances by GSH is catalyzed by a group of cytosolic enzymes, GST. The capacity of GSH to act as a reductant is prompted by the GPx that converts toxic peroxides to less toxic hydroxyl derivatives. In this reaction, GSH itself is oxidized to form GSSG. GSSG is again reduced to GSH by another enzyme, GR, which utilizes NADPH. In our present study, GSH levels were decreased and GSSG levels were increased with the increasing duration of VSSA treatment. The decreased GSH levels represent its increased utilization with the increasing time interval of VSSA treatment. 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 GR to transform GSSG to GSH, due to the increasing production of ROS at a rate that exceeds the ability to regenerate GSH in macrophages with increasing time of VSSA treatment.

In this study, significant elevation of nitrate generation and MPO activity in macrophage were observed maximally in VSSA treated group at 3 h. Hypochlorous acid is generated in the presence of MPO and initiates the deactivation of antiproteases and the activation of latent proteases and leads to the cellular damage. Antioxidant enzymes are considered to be a primary defense that prevents biological macromolecules from oxidative damage. SOD rapidly dismutates superoxide anion to less dangerous H_2O_2, which is further degraded by CAT and GPx to water and oxygen[15]. The results of the present study showed a significant maximal fall in SOD and CAT activities in macrophage of VSSA treated group at 3 h. SOD, dismutate superoxide anion and the same in turn is a potent inhibitor of CAT[18]. The depletion in SOD activity was may be due to dispose of the free radicals, produced due to VSSA 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.

In our present study, the increasing levels of GSSG and decreasing GR activity along with increasing duration of VSSA treatment may hold up the explanation. GPx activity was also decreased in time dependent manner during VSSA treatment in macrophage may be due to less availability of substrate, as GSH level was decreased time dependent manner. GPx works non-specifically to scavenge and decompose excess hydro peroxides including H_2O_2, which may be prevalent under oxidative stress[11,12]. GST mainly detoxifies electrophilic compounds[13], 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 decreasing level of GSH and decreased activity of GSH-dependent enzymes, *i.e.* GPx, GR, and GST in peritoneal macrophages on *in vitro* treatment with increasing time of VSSA treatment (1 h to 24 h) may be due to increased utilization to scavenge the free radical generation. But it was observed that duration of the VSSA treatment was higher than 3 h, the scope of further utilization of these enzymes remains the same. A decrease in the level of antioxidant status can lead to the excessive availability of super oxide and peroxyl radicals which in turn generate hydroxyl radicals, resulting in the initiation and propagation of lipid peroxidation during VSSA treatment.

Lipid peroxidation is known to cause cellular injury by inactivation of membrane enzymes and receptors, depolymerisation of polysaccharide, as well as protein cross-linking and fragmentation. Since membrane lipids are vital for the maintenance and integrity of cell function, the breakdown of membrane phospholipids and lipid peroxidation due to the generation of free radicals are expected to change membrane structure, fluidity, transport and antigenic properties, all of which play an important role in the pathogenesis of organ disorders[18]. PC 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[17], as evidenced by the decreased activity of antioxidant enzymes like SOD, CAT, GPx, GR, and GST.

In summery, the present study clearly established that VSSA can time dependently increase the superoxide anion generation through the activation of NADPH oxidase in murine peritoneal macrophages, which reduce the important antioxidant status.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

This work reports on pathophysiological aspect on an important bacterial infection. The work is on the clinical chemistry parameter focusing on oxidation reduction phenomenon. The work contains some interesting informations from little studied area.

Research frontiers

This work has some novelties and reports on interesting clinical chemistry study that link to the clinical microbiology work on *S. aureus*. It is a good attempts and up-to-date investigation in the era of worldwide studies on antioxidation science.

Related reports

This work reports on the area that lack for information in clinical chemistry. Although the antioxidant is widely mentioned it is limitedly reported in the infection by *S. aureus* pathogen. Few reports are available on this aspect.

Innovations and breakthroughs

This work has novelty and reports on rarely published *S. aureus*. This is a good paper in clinical pathology, microbiology and clinical chemistry. The authors successfully trace the area that has limitedly studied in the previous reports of *S. aureus* studies.

Applications

This work can be further applied in the field of pathophysiology and microbiology. It can be further referenced in future studies on pathology of bacterial infection as well as the investigation in pharmacological study.

Peer review

This work is interesting and contains novel data. This work can be future referenced in the field of microbiology and tropical medicine. Its eminent finding is in the clinical chemistry finding on the antioxidant assessment and corresponding bacterial pathogen.

Reference

[1] Das D, Saha SS, Bishayi B. Intracellular survival of *Staphylococcus aureus*: correlating production of catalase and superoxide dismutase with levels of inflammatory cytokines. *Inflamm Res* 2008; **57**: 340-349.

[2] Beaman L, Beaman BL. The role of oxygen and its derivatives in microbial pathogenesis and host defense. *Ann Rev Microbiol* 1984; **38**: 27-48.

[3] Mahapatra SK, Chakraborty SP, Das S, Roy S. Methanol extract of *Ocimum gratissimum* protects murine peritoneal macrophages from nicotine toxicity by decreasing free radical generation, lipid and protein damage, and enhances antioxidant protection. *Oxid Med Cell Longev* 2009; **2**(4): 222-230.

[4] Chakraborty SP, Mahapatra SK, Sahu SK, Chattopadhyay S, Pramanik P, Roy S. Nitric oxide mediated *Staphylococcus aureus* pathogenesis and protective role of nanoconjugated vancomycin. *Asian Pac J Trop Biomed* 2011; **1**(2): 102-109.

[5] Chakraborty SP, Mahapatra SK, Bal M, Roy S. Isolation and identification of vancomycin resistant *Staphylococcus aureus* from post operative pus sample. *Al Ameen J Med Sci* 2011; **4**(2): 152-168.

[6] Mahapatra SK, Das S, Bhattacharjee S, Gautam N, Majumdar S, Roy S. *In vitro* nicotine-induced oxidative stress in mice peritoneal macrophages: a dose-dependent approach. *Toxicol Mech Methods* 2009; **19**: 100-108.

[7] Oliveros LB, Videla AM, Gimenez MS. Effect of dietary fat saturation on lipid metabolism, arachidonic acid turnover and peritoneal macrophage oxidative stress in mice. *Braz J Med Biol Res* 2004; **37**: 311-320.

[8] Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP. Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *J Immunol* 2000; **164**: 3713-3722.

[9] Ives TJ, Schwab UE, Ward ES, Hall IH. *In-vitro* anti-inflammatory and immunomodulatory effects of grepafloxacin in zymogen A- or *Staphylococcus aureus*-stimulated human THP-1 monocytes. *J Infect Chemother* 2003; **9**: 134-143.

[10] Sandhu SK, Kaur G. Alterations in oxidative stress scavenger system in aging rat brain and lymphocytes. *Biogerontology* 2002; **3**: 161-173.

[11] Chakraborty SP, Mahapatra SK, Roy S. *In vitro* time-dependent vancomycin-resistant *Staphylococcus aureus*-induced free radical generation and status of antioxidant enzymes in murine peritoneal macrophage. *Toxicol Mech Methods* 2012; **22**(1): 9-22.

[12] Chakraborty SP, Kar Mahapatra S, Sahu SK, Das S, Tripathy S, Dash S, et al. Internalization of *Staphylococcus aureus* in lymphocytes induces oxidative stress and DNA fragmentation: possible ameliorative role of nanoconjugated vancomycin. *Oxid Med Cell Longev* 2011; doi: 10.1155/2011/942123.

[13] Chakraborty SP, Pramanik P, Roy S. Protective role of nanoconjugated vancomycin against vancomycin sensitive *Staphylococcus aureus* induced oxidative stress and DNA damage. *Int J Pharm Sci Res* 2012; **3**(2): 405-415.

[14] Chakraborty SP, Das S, Chattopadhyay S, Tripathy S, Dash SK, Pramanik P, et al. *Staphylococcus aureus* infection induced redox signaling and DNA fragmentation in T-lymphocytes: possible ameliorative role of nanoconjugated vancomycin. *Toxicol Mech Methods* 2012; **22**(3): 193-204.

[15] Chakraborty SP, Das S, Pramanik P, Roy S. Vancomycin resistant *Staphylococcus aureus* induced oxidative stress in liver and kidney: protective role of nanoconjugated vancomycin. *Int J Life Sci Pharm Res* 2011; **1**(1): L60-L74.

[16] Chakraborty SP, Mahapatra SK, Sahu SK, Pramanik P, Roy S. Antioxidative effect of folate-modified chitosan nanoparticles. *Asian Pac J Trop Biomed* 2011; **1**(1): 29-38.

[17] Kar Mahapatra S, Chakraborty SP, Majumdar S, Bag BG, Roy S. Eugenol protects nicotine-induced superoxide mediated oxidative damage in murine peritoneal macrophages *in vitro*. *Eur J Pharmacol* 2009; **623**(1-3): 132-140.

[18] Kar Mahapatra S, Bhattacharjee S, Chakraborty SP, Majumdar S, Roy S. Alteration of immune functions and Th1/Th2 cytokines balance in nicotine-induced murine macrophages: immunomodulatory role of eugenol and N-acetylcysteine. *Int Immunopharmacol* 2011; **11**(4): 485-495.