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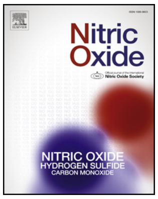

Reactive nitrogen species induced catalases promote a novel nitrosative stress tolerance mechanism in *Vibrio cholerae*

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ABSTRACT

Vibrio cholerae faces nitrosative stress during successful colonization in intestine. Very little information is available on the nitrosative stress protective mechanisms of *V. cholerae*. Reports show that NorR regulon control two genes *hmpA* and *nnrS* responsible for nitric oxide (NO) detoxification in *V. cholerae*. In the present study we first time report a novel role of *V. cholerae* catalases under nitrosative stress. Using zymogram analysis of catalase we showed that KatB and KatG activity were induced within 30 min in *V. cholerae* in the presence of sodium nitroprusside (SNP), a NO donor compound. Surprisingly, *V. cholerae* cell survival was found to be decreased under nitrosative stress if catalase activities were blocked by ATz, a catalase inhibitor. Flow cytometry study was conducted to detect reactive oxygen species (ROS) and reactive nitrogen species (RNS) using DHE and DHR123, fluorescent probes respectively. Short exposure of SNP to *V. cholerae* did not generate ROS but RNS was detectable within 30 min. Total glutathione content was increased in *V. cholerae* cells under nitrosative stress. Furthermore, Superoxide dismutase (SOD) and Glutathione reductase (GR) activities remained unchanged under nitrosative stress in *V. cholerae* indicated antioxidant role of NO which could produce peroxynitrite. To investigate the role of catalase induction under nitrosative stress in *V. cholerae*, we conducted peroxynitrite reductase assay using cell lysates. Interestingly, SNP treated *V. cholerae* cell lysates showed lowest DHR123 oxidation compared to the control set. The extent of DHR123 oxidation was more in *V. cholerae* cell lysate when catalases were blocked by ATz.

1. Introduction

Vibrio cholerae, the causative agent of the disease cholera pass through the stomach and colonize the small intestines of its host. Symptomatic *V. cholerae* infection induces Nitric oxide (NO) production in humans [1,2]. *V. cholerae* faces many challenges during successful colonization of the intestines. NO either generated by host inducible nitric oxide synthase (iNOS) activity or derived from acidified nitrite in the stomach are deleterious for *V. cholerae* [2,3]. Due to its very short half-life, NO quickly oxidize to generate nitrite (NO_2), nitrate (NO_3), dinitrogen trioxide (N_2O_3) etc. NO reacts with cellular iron to form dinitrosyl iron complexes (DNIC) [4]. NO also reacts with superoxide to form peroxynitrite ($ONOO^{\cdot}$) [5]. NO can also form S-nitrosogutathione (GSNO) with cellular glutathiones [6]. Peroxynitrite ($ONOO^{\cdot}$), one of the most toxic reactive nitrogen species (RNS), is mainly generated by the addition of superoxide anion ($O_2^{\,-}$) and nitric oxide radical ($NO^{\cdot}$) at near diffusion-controlled rate of $1.9 \times 10^{10}\,M^{-1}\,s^{-1}$ [7]. Cellular proteins, lipids and DNA are the key targets of these reactive nitrogen species (RNS) [8]. Excess production of these NO and RNS alter the cellular redox status and causes stress which is termed as nitrosative stress [9–14]. The mechanism that protects *V. cholerae* cells against the deleterious effects of nitrosative stress remains to be elucidated and is a topic of ongoing research [15].

Studies showed that *V. cholerae* strains deficient in DNA repair or reactive nitrogen species defense are defective in intestinal colonization [16]. It has been demonstrated that the regulatory protein NorR regulates the expression of NO detoxification genes *hmpA* and *nnrS* of *V. cholerae* under microaerobic conditions in vitro [17]. A metabolomic screen on NO-treated and -untreated bacteria identified the function of NnrS which is particularly important for resistance to nitrosative stress under anaerobic conditions [18]. We first time showed the presence of GSNO reductase activity for the detoxification of GSNO in *V. cholerae* which was previously identified in *Escherichia coli*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis*. Interestingly,

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GSNO reductase activity of *V. cholerae* was regulated by posttranslational modification through S-nitrosylation under nitrosative stress which could be reversed by dithiothreitol (DTT) [15]. *V. cholerae* also encounters reactive oxygen species (ROS) and the transcriptional regulator OxyR in *V. cholerae* is critical for antioxidant defense [19,20]. *V. cholerae* can produce two distinct catalases, KatB and KatG, which contribute to ROS homeostasis [21]. Furthermore, two catalases, KatG and KatB, either when expressed in living cells, present in culture supernatants, or added as purified recombinant proteins, could rescue the growth defect in *oxyR* mutant strains of *V. cholerae.* Interestingly, it could colonize infant mouse intestines similar to that of wild type [22]. So, two catalases, KatG and KatB are indispensable for successful colonization of *V. cholerae.* In the present study to investigate the role of catalases in *V. cholerae* under nitrosative stress we identified a novel stress response mechanism in which NO can induce both KatG and KatB and this induction of catalases are RNS dependent. Moreover, our *in vitro* studies support the role of catalase in protection against peroxynitrite mediated oxidation.

2. Material and methods

2.1. Strains and media

Three clinical strains of *Vibrio cholerae* which were used in this study: PC2 (non O1 non O139 serotype), NB2 (O1 serotype) and SG24 (O139 serotype). Those strains were generously provided by Dr. Prasanta Kumar Bag, Professor, Dept. of Biochemistry, University of Calcutta. Strains were grown in Luria Bertani broth (LB) supplemented with 1% NaCl and pH was adjusted at 7.5. *katB/katG* double mutant of *V. cholerae* (El tor C6706) was a generous gift from Prof. Jun Zhu's laboratory, Department of Microbiology, Nanjing Agricultural University, Nanjing, Jiangsu, China. Δ*katB/katG* strain was grown similarly but in the presence of streptomycin (100 μg/ml).

2.2. Preparation of sodium nitroprusside (SNP), 3-amino-1,2,4-triazole (ATz), S-Nitrosoglutathione (GSNO), hydrogen peroxide (H_2O_2) and peroxynitrite

Sodium nitroprusside (SNP) stock solution was prepared by weighing appropriate amount of SNP and dissolving it in sterile mili-Q water. 3-Amino-1,2,4-triazole stock solution was prepared in similar way. In case of H_2O_2 particular volume of stock H_2O_2 was mixed with milli-Q water to make working stock solution. For making GSNO, equal volume of 1 M GSH (in 1 N HCl) and 1 M $NaNO_2$ (dissolved in milli-Q water) were mixed at dark and kept at cold followed by spectrum analysis and concentration determination [9]. GSNO concentration was calculated using $€_{335\ \mathrm{nm}} = 586\,M^{-1}\,cm^{-1}$. All solutions were finally passed through 0.22 μm filter to make them sterile. For preparation of peroxynitrite, a 2 M solution of H_2O_2 in 2 N NaOH, 2 mM EDTA was prepared and stirred vigorously with amyl nitrite (4:1, V/V) for 3 h on ice. The aqueous layer was separated. It was then washed thrice with ice-cold hexane. Finally the aqueous layer was stirred with activated MnO_2 for 30 min on ice. It was aliquoted and stored at 80 °C. Peroxynitrite concentration was calculated using extinction coefficient 302 nm = $1760\,M^{-1}\,cm^{-1}$ [23].

2.3. Exposure to SNP, GSNO, H_2O_2 and ATz

PC2, NB2 and SG24 cells were grown overnight in separate test tubes and were diluted individually into conical flasks containing fresh LB media to reach 0.05 O.D. at 600 nm. The cultures were then incubated at 37 °C at shaking condition until the O.D. reached 0.5. Each culture was then distributed into two conical flasks equally for the SNP treatment. Each culture was treated with 1 mM SNP keeping another culture set as control where decomposed SNP was added. All the cultures were then incubated for 2 h. Similar procedure was applied for NB2 strain of *V. cholerae* to treat with GSNO and H_2O_2 and cultures were incubated for 30 min and 2 h along with control set. Finally, after incubation the cells were lysed and crude cell lysates were kept and stored at −80 °C for further use.

For ATz (3-Amino-1,2,4-triazole) treatment to inhibit catalase activity, overnight grown NB2 culture was diluted in fresh LB media to make O. D. at 600 nm 0.05 and then incubated at 37 °C until cells reach mid log phase (0.5 O.D.). Then cultures were distributed in 6 conical flasks equally and treatments were done of 1 mM SNP, 1 mM & 3 mM GSNO and 0.5 mM & 1 mM H_2O_2. Then cultures were incubated for 30 min and 2 h along with control set. Finally after incubation the cells were lysed and crude cell lysates were kept and stored at −80 °C for further use.

2.4. Flow cytometry

V. cholerae strain NB2 was grown overnight in LB media under shaking condition (140 rpm) at 37 °C. Overnight grown culture was then inoculated to a fresh sterile LB medium making the O.D. at 600 nm 0.05 and distributed into four sterile conicals. 3-Amino-1,2,4-triazole (ATz) was added to two individual conical flasks containing cultures making the final concentration 50 mM ATz and incubated at 37 °C under shaking condition until the O. D. of all cultures at 600 nm reached 0.5. SNP was added to two individual cultures, one of which is already pretreated with 50 mM ATz, making the final concentration of 1 mM SNP. All cultures were then incubated for either 5 or 15 or 30 min at 37 °C under shaking condition along with a control set for each time point and centrifuged at 5000 rpm for 10 min in a cold centrifuge. Cell pellets were resuspended and washed with 10 mM PBS (pH 7.4). The washing step was followed by centrifugation at 5000 rpm for 10 min and cell pellets were collected. Cell pellets were resuspended in 400 μl PBS of pH 7.4 in one set and in other set cell pellets were resuspended in 10 mM Tris-HCl (pH-7.5) buffer. Dihydrorhodamine 123 (DHR 123) and Dihydroethidium (DHE) were added to PBS containing culture tube and Tris-HCl containing culture tube respectively to make a final concentration of 10 μM DHR 123 and 50 μM DHE. The samples were then incubated for 15 min at room temperature in dark. After incubation culture tubes were centrifuged at 5000 rpm and supernatants were discarded. The pellets were washed twice with respected buffers followed by centrifugation. Finally, the cells were resuspended in respected buffers and were subjected to flow cytometry analysis to monitor for RNS and ROS. Δ*katB/katG* strain was grown similarly like NB2 strain but only treated with 1 mM SNP along with their control set following similar centrifugation, washing and dye treatments for flow Cytometry. The DHR fluorescence was measured through the FITC-channel, and DHE fluorescence was determined using the propidium iodide-channel. The photomultiplier tube (PMT) voltage was kept at 193 mV for FITC-channel and 224 mV for PI-channel, and the FACS flow rate was constant at 1 ($10\,\mu l\,min^{-1}$) in all experiments. Ten thousand events were recorded for each sample. Histograms were generated by plotting the bacterial count against fluorescence in the respective channel. The data were analyzed by specific population gating depending on the subset for each experiment and were kept constant for a particular assay. The gating analysis was done using FACS Diva software.

Similar treatment procedure was followed for zymogram analysis and enzymatic assays where cultures were harvested at 5000 rpm in a cold centrifuge for 10 min and supernatants were discarded. Cell pellets were washed with 10 mM PBS (pH 7.4) and stored at −80 °C.

2.5. Crude cell lysates preparation

Cell pellets were resuspended in 20 mM Tris-HCl of pH 7.5 (with 1 mM EDTA, 1 mM PMSF) and lysed by sonication. Lysed cells were then centrifuged at 12000 rpm for 10 min at 4 °C and supernatants were collected. Collected supernatants were stored at −80 °C in different aliquots for further use. Total protein was determined by Bradford's method [24].

2.6. Detection of catalase activity by zymogram analysis

Catalase activities in protein samples were detected by zymogram analysis protocol, reported by Weydert et al. [25] with some modifications. A 1.5 mm thick native-PAGE gel was prepared with 5% stacking and 8% resolving gels. Protein samples were prepared without adding SDS and β-mercaptoethanol to maintain the native conformation of proteins. Prepared and normalized protein (40 μg) were then loaded in individual wells of native gel along with 5 μg catalase protein as positive control in one well. Electrophoresis was done with 30 mA steady current at 4 °C and carefully observed. When the dye front reached the bottom of the gel, the gel was electrophoresed further for 1.5 h more and then the electrophoresis was stopped. The gel was removed from the glass plates and placed in a glass staining dish. Then the gel was washed with double distilled water for 5 min in shaking condition and the step was repeated for 2 times. After the washing step, 0.003% H_2O_2 solution (vol/vol) was freshly prepared. Then the gel was incubated in 0.003% H_2O_2 solution for 10 min in shaking condition. After this step the gel was rinsed with double distilled water twice for 5 min. Simultaneously 2% Ferric chloride ($FeCl_3$) and 2% Potassium ferricyanide {K_3 [Fe $(CN)_6$]} solution was prepared in equal volume separately in different glass conical for staining the gel. Finally, the two stains were poured together directly on the gel and was placed in a shaker carefully. When the achromatic gel bands of catalase appeared, the stain solution was poured off and gel was rinsed extensively with double distilled H_2O. The gel picture was taken by Gel Doc™ XR+ from BIO RAD imaging machine and fold change of catalase activity was measured by densitometric analysis of bands.

2.7. Enzyme activity assay

Activity of Catalase in crude cell free extracts was measured by observing the decreasing absorbance of H_2O_2 at 240 nm, due to catalase mediated H_2O_2 degradation in reaction mixture [26].

Activity of Superoxide dismutase (SOD) in cell free extracts was measured spectrophotometrically by the modified method established by Beauchamp & Fridovich in 1971 [27], where, formation of purple diformazan dye from the reaction of Nitroblue tetrazolium (NBT) and superoxides was monitored at 560 nm. Where Xanthine (100 μM) and Xanthine oxidase (0.002U/μl) is used as superoxide generator and NBT is used as chromogen in presence of superoxides. The higher the activity of SOD, lower the extent of formazan dye formation as SOD competes with NBT for generated superoxides. 1 Unit of SOD activity is referred as amount of SOD responsible for 50% inhibition of formazan dye formation rate of 0.020 ± 0 .005 O.D. at 560 nm/min.

The Glutathione reductase (GR) assay was done in 50 mM K-phosphate (pH −7.0) buffer containing 1 mM EDTA, 1 mM GSSG, 0.15 mM NADPH, in a total volume of 500 μl. GR activity of crude cell free extracts was determined by measuring the decrease in absorbance at 340 nm [28].

Peroxynitrite reductase assay was done using peroxynitrite-mediated oxidation of DHR 123 to rhodamine 123 as previously described [29]. Various concentrations of cell lysate or BSA (negative control) were incubated in potassium phosphate buffer (pH 7.2), 100 μM EDTA and 100 μM dihydrorhodamine 123 (DHR 123). Peroxynitrite was added to a final concentration of 20 μM, then agitated vigorously for 1 min and formation of Rhodamine 123 was monitored spectrophotometrically at 500 nm.

2.8. Viable count determination of V. cholerae

For viable count determination, cultures were taken aseptically from the control and treated set, serially diluted and were spreaded on LA plates. Plates were kept at 37 °C overnight. Next day the colonies grown on plates for individual treatments along with control set were counted and cell viability was determined in respect to their control plate.

2.9. Total glutathione and GSSG content

These parameters were measured according to the method described by Akerboom *et al.* [30]. In brief, freshly prepared, crude cell-free extract was added to equal volume of 2 M $HClO_4$ containing, 2 mM EDTA and incubated on ice for 10 min. The mixture was centrifuged at 5000 × *g* for 5 min. Resulting supernatant was neutralized with 2 M KOH containing 0.3 M HEPES. After centrifugation at 5000 × *g* for 5 min, the neutralized supernatant was used for estimation of the above-mentioned parameters. Total glutathione was estimated following Glutathione Reductase (GR)-dependent DTNB reduction assay spectrophotometrically measuring 5-thio-2-nitrobenzoate (TNB) formation at 412 nm. The reaction mixture contained 100 mM K-phosphate, pH 7.0, 1 mM EDTA, 0.12 U GR, 0.2 mM NADPH, 0.063 mM DTNB, and sample in a total volume of 500 μl. Same neutralized extract was treated with 2-vinylpyridine (50:1, v/v) for 1 h at room temperature. Then, it was used for GSSG estimation using the above method. Reduced Glutathione content was determined from the difference between the total and oxidized glutathione content of the sample.

2.10. Statistical analysis

All experimental results were expressed as mean ± SD, for n = 3. The statistical evaluation was performed with two-way ANOVA followed by two tailed paired Student's t-test; p value ≤ 0.05 or 0.01 was considered significant.

3. Results

3.1. Induction of catalase activity in non-O1, O1, and O139 strains of V. cholerae under nitrosative stress

In our previous study, three clinical strains of *V. cholerae* were tested for their nitrosative stress response pattern towards sodium nitroprusside (SNP) and S-Nitrosoglutathione (GSNO). Among them, *V. cholerae*, belonging to both O1 and O139 serotypes, showed moderate resistance to SNP and GSNO. However, a *V. cholerae* strain belonging to non O1 and non O139 showed sensitivity to SNP but resistance towards GSNO [15]. Several enzymes are found to be important components of cellular defense system against reactive nitrogen species (RNS) through genetic studies in bacteria and yeasts. So, to investigate the effect of nitrosative stress on *V. cholerae*, we tested the induction of catalase using an in-gel activity assay in three strains of *V. cholerae*, strains NB2, SG24, and PC2 belonging to O1, O139 and non-O1, and non- O139, respectively, following 2 h stress treatment in the presence of 1 mM SNP. For this, exponential phase whole cell lysates were subjected to non-denaturing PAGE followed by in-gel catalase activity staining [24]. Purified bovine catalase was used as a positive control in the activity staining of the catalase. Two catalase activity region (I and II) exhibiting distinct migration patterns were detected in gel lanes corresponding to NB2, SG24, and PC2 belonging to O1, O139 and non-O1, and non- O139 exponential phase cells (Fig. 1). As all the strains of *V. cholerae* have potential to produce two catalases, one slow moving and another fast-moving band might correspond to KatG and KatB. In a similar in-gel catalase activity staining in *V. cholerae* O1, it has been proved by Mass Spectrometric analysis that fast-moving band is KatG and slow-moving band is KatB [21]. Interestingly, all the strains belonging to O1, O139 and non-O1, and non-O139 exponential phase cells produced significantly higher KatB level upon nitrosative stress in the presence of 1 mM SNP for 2 h compared to the control set where decomposed SNP was used. KatG activity was found to be decreased upon SNP treatment

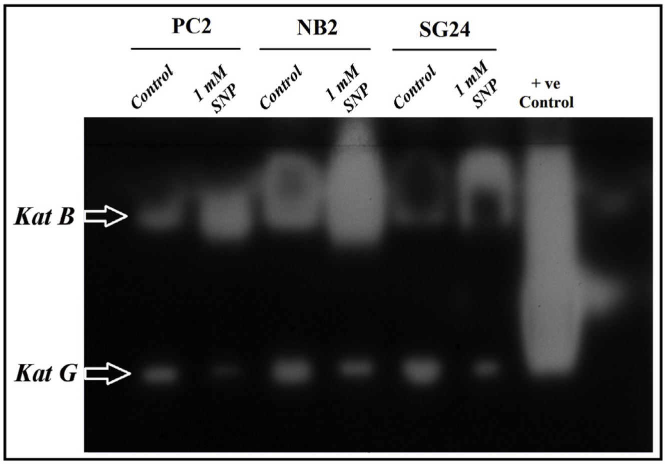

Fig. 1. Zymogram analysis of Catalase of *V. cholerae* strains PC2, NB2 and SG24 upon 1 mM SNP treatment for 2 h. *V. cholerae* strains PC2, NB2 and SG24 were treated with 1 mM SNP for 2 h along with their control sets, where decomposed SNP was used. Cell lysates were prepared and subjected to zymogram analysis for catalase activity.

for 2 h in all these strains subjected to in-gel catalase activity staining. As *V. cholerae* strain NB2 belonging to O1 showed maximum induction of KatB activity in the presence of 1 mM SNP for 2 h, further experiments were conducted using this strain.

3.2. Time dependent induction of catalase activity under nitrosative stress in NB2 strain of V. cholerae

To determine time dependent induction of catalase activity under nitrosative stress in NB2 strain of *V. cholerae*, three log phase cultures of NB2 strain (0.5 O.D.) were grown in the presence of 1 mM SNP for 5, 15 and 30 min respectively along with their controls. Whole cell lysate was prepared from each time point and each cell lysate was subjected to non-denaturing PAGE followed by in-gel catalase activity staining (Fig. 2). Purified bovine catalase was used as a positive control in the activity staining of the catalase. There was no change in KatB and KatG activity profile in 5 min time point between 1 mM SNP treated and the control set of NB2 strain of *V. cholerae.* Interestingly, activities of KatG and KatB of NB2 strain of *V. cholerae* were significantly increased in 15 min treatment with 1 mM SNP compared to the control. Activity of KatG and KatB was further increased following 1 mM SNP treatment for 30 min in NB2 strain of *V. cholerae* compared to the control. So, it can be concluded that both KatG and KatB activity were increased under nitrosative stress following 1 mM SNP treatment in NB2 strain of *V. cholerae*.

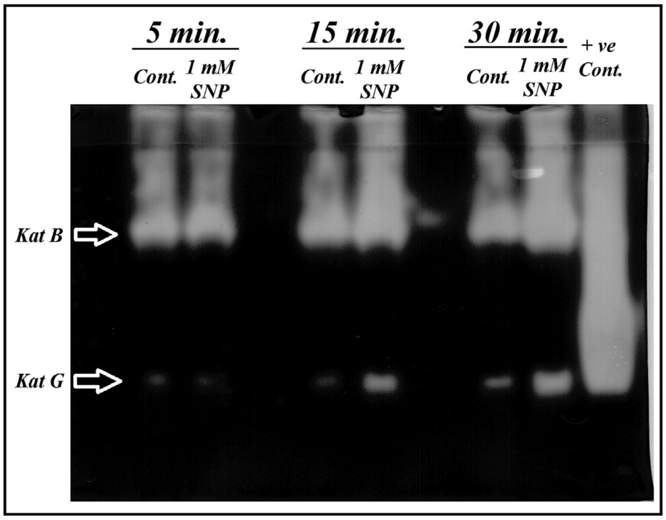

Fig. 2. Zymogram of time dependent induction of catalase in *V. cholerae,* NB2 strain in presence of 1 mM SNP.

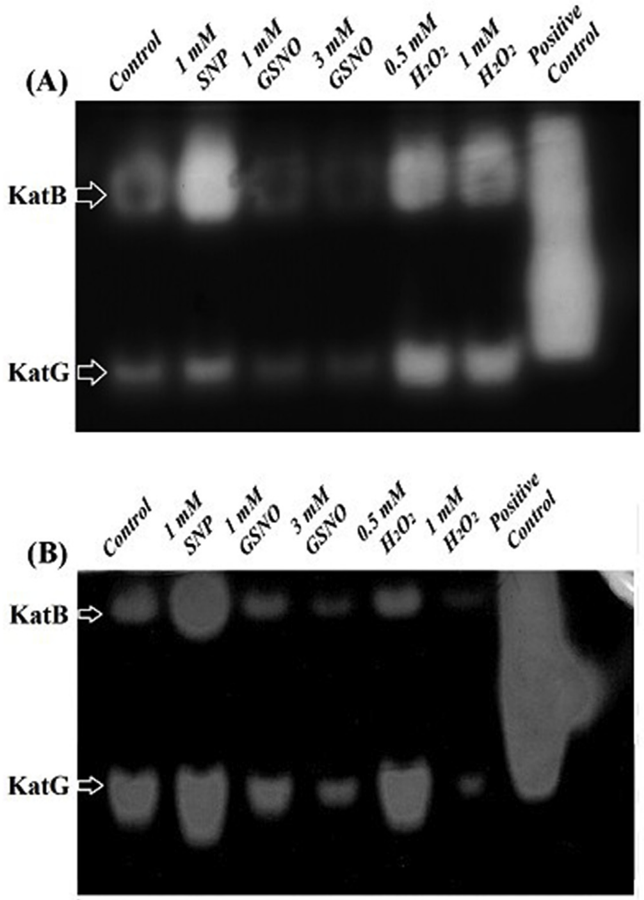

Fig. 3. Zymogram analysis of catalase of *V. cholerae,* NB2 strain upon treatment with different NO donors along with graded concentrations of H_2O_2 (A) 30 min and (B) 2 h.

3.3. Induction of catalase activity was maximum for SNP treatment in NB2 strain of V. cholerae

To examine the extent of induction of Catalase activity under nitrosative stress in NB2 strain of *V. cholerae*, different NO donor compounds were used for 30 min and 2 h time of incubation. Among the different NO and RNS donors used, SNP treatment showed maximum induction of KatB and KatG activity (Fig. 3A and B). However, no induction of KatB and KatG activity was observed following 1 and 3 mM GSNO treatment for 30 min as well as in 2 h time of incubation compared to control. Significant induction of both KatB and KatG activity was observed in NB2 strain of *V. cholerae* under treatment of 0.5 mM H_2O_2 at both the time point (Fig. 3A). However, both KatB and KatG activities of NB2 strain were decreased significantly following 1 mM H_2O_2 treatment for 2 h (Fig. 3B).

3.4. Catalase activity was blocked by ATz in NB2 strain of V. cholerae

To examine whether the catalase activity of NB2 strain of *V. cholerae* is inhibited by ATz or not, four sets of experiments were conducted. The initial inoculum was divided into four conical flasks in such a way so that each flask contained 0.05 O.D. cells of NB2. One flask was marked

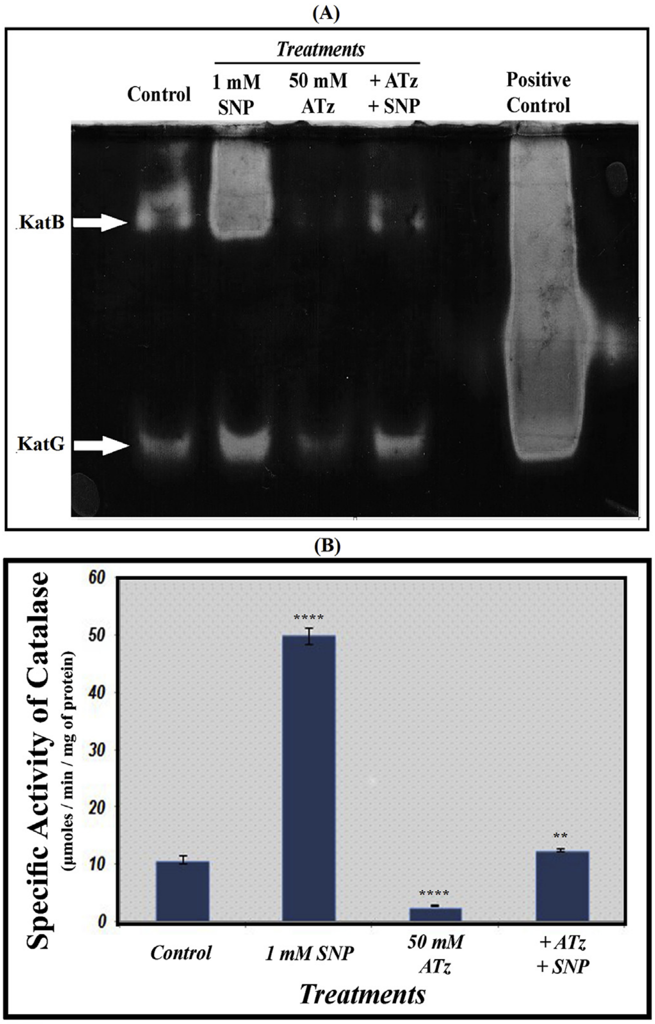

Fig. 4. (A) Zymogram analysis and (B) specific activity of catalase of NB2 strain of *V. cholerae* in presence of catalase inhibitor ATz (3-amino-1,2,4-triazole) and NO donor SNP. This data is represented as mean ± SD (n = 3). **p < 0.01, ****p < 0.0001.

as control. In two flasks, ATz was added to a final concentration of 50 mM. In another flask only 0.05 O.D. cells were taken. All the flasks containing cultures of NB2 strain of *V. cholerae* were allowed to grow until 0.5 O.D. was reached. Then two cultures of NB2 strain of *V. cholerae* were treated with 1 mM SNP among which one culture was already pretreated with ATz. All the cultures were incubated for 30 min followed by cell lysate preparation. Lysates prepared from all the respective cultures were subjected to zymogram analysis of catalase activity staining (Fig. 4A) and specific activity determination (Fig. 4B). There was almost complete inhibition of catalase activity in ATz pretreated culture. Similar observation was noted for SNP treated cells of NB2 which was previously pretreated with ATz. But induction of catalase activity (~2 fold) was observed in NB2 culture which was only treated with 1 mM SNP. Specific activity data (~5 fold increase) from the same culture corroborated well with the zymogram analysis. Thus, in conclusion, catalase activity was induced in NB2 strain of *V. cholerae* under nitrosative stress and it can be inhibited by ATz.

3.5. Catalases protect V. cholerae under nitrosative stress

To evaluate the role of catalase under nitrosative stress in *V. cholerae*, cell viability was checked under different conditions (Fig. 5). Interestingly, cell viability was almost ~97% for NB2 strain of *V. cholerae* treated with 1 mM SNP compared to the control indicating *V. cholerae* cells were able to withstand nitrosative stress up to that concentration as previously reported (15). We wanted to check the cell viability of the *V. cholerae* cultures pretreated with catalase inhibitor as 50 mM final concentrations of Atz could block catalase activity determined by zymogram analysis as well as in spectrophotometric assay. There were almost 87% cells of NB2 remained viable following 50 mM ATz treatment indicating inhibition of catalase did not affect *V. cholerae* cell viability significantly. If catalase promotes nitrosative stress tolerance then pretreatment of *V. cholerae* culture with catalase inhibitor followed by SNP treatment might affect cell viability. In fact contribution of catalase in promoting nitrosative stress tolerance was reflected in decreased cell viability (~68%) of NB2 cells of *V. cholerae* pretreated with 50 mM ATz followed by 1 mM SNP treatment. When Δ*katB/katG* strain was exposed to 1 mM SNP treatment, similar result was obtained with respect to untreated control set of mutant where cell viability was reduced (~65%). Decrease in Cell viability of *V. choleare* upon nitrosative stress in ATz mediated catalase inhibition or in Δ*katB/katG* strain reflects a similar impact. Thus, it can be concluded that catalases protect *V. cholerae* cells under nitrosative stress.

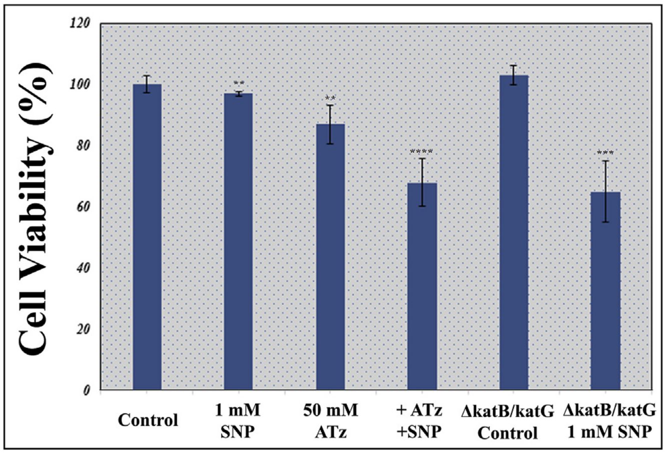

Fig. 5. Cell viability determination of *V. cholerae* NB2 strain and *katB/katG* double mutant along with their treated sets. The cell viability is expressed as percentage of CFU/ml with respect to the Control set. CFU counts were done in technical as well as biological triplicates and represented considering the mean value with ± SD, n = 3. **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.6. FACS analysis revealed only RNS generation under nitrosative stress in V. cholerae

Previously it has been shown that catalases promote resistance to oxidative stress in *V. cholerae*. To determine the ROS and RNS generation status in NB2 strain and Δ*katB/katG* strain of *V. cholerae* under nitrosative stress we conducted FACS analysis using fluorescence probe DHE and DHR. Dihydroethidium (hydroethidine or DHE) is a widely used ethidium-based, redox-sensitive fluorescent probe. DHE has been shown to be oxidized by superoxide to form 2-hydroxyethidium or by non-specific oxidation by other sources of reactive oxygen species (ROS) to form ethidium [31]. Dihydrorhodamine 123 (DHR) is the most frequently used probe for measuring $ONOO^-$ [32]. This assay is based on the oxidative conversion of DHR123 to its corresponding two-electron oxidized fluorescent product, rhodamine. Results showed that control NB2 strain and Δ*katB/katG* strain of *V. cholerae* cells did not produce reactive nitrogen species (RNS) or reactive oxygen species (ROS) either in 5 min or 30 min incubation period (Fig. 6A–H). In NB2 strain (Fig. 6a), upon treatment of ATz, a catalase inhibitor, a fraction of cell population (12 %) did accumulate RNS immediately (5 min) as indicated by the DHR staining. But this fraction was absent at later stages of treatment (30 min). Separately, on treatment of the RNS

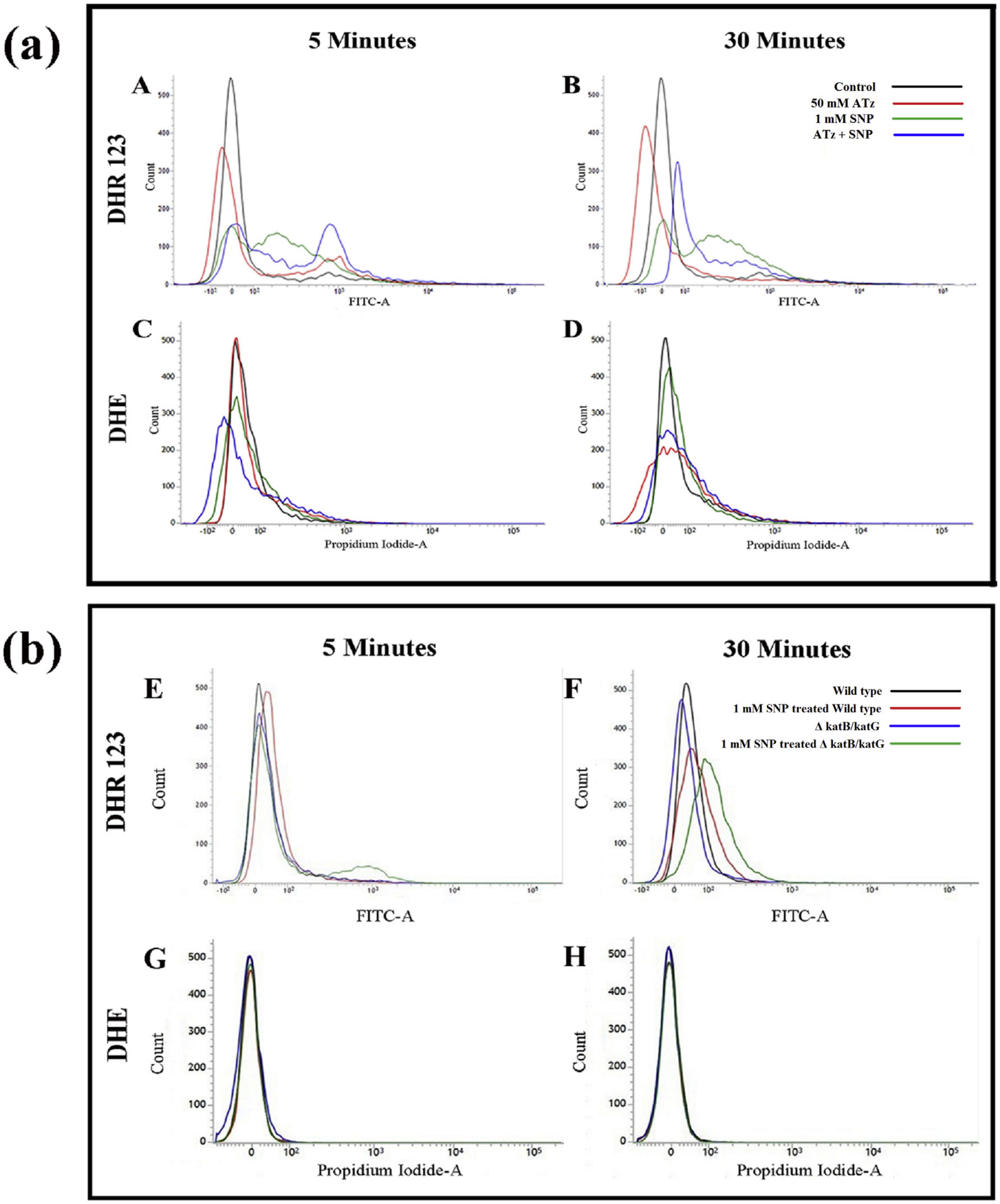

Fig. 6. Flow Cytometry analysis of differential RNS and ROS generation in *Vibrio cholerae* (NB2 and katB/katG double mutant) cells using Dihydrorhodamine 123 (DHR123) and Dihydroethidium (DHE) respectively. (**a**)The histogram shows the FACS analysis of *Vibrio cholerae,* NB2 cell population treated with ATz, SNP or both for 5 min (A and C) and 30 min (B and D). The cells were stained either with DHR 123 (A and B) for RNS detection or DHE (C and D) for ROS detection. Black line = Control, Red line = 50 mM ATz treated, Green line = 1 mM SNP treated, Blue line = 50 mM ATz pretreated + 1 mM SNP treated. (**b**) The histogram shows the FACS analysis of *Vibrio cholerae*, Wild Type and *katB/katG* double mutant cell population treated with only 1 mM SNP for 5 min (E and G) and 30 min (F and H). The cells were stained either with DHR 123 (E and F) for RNS detection or DHE (G and H) for ROS detection. Black line = Wild Type Control, Red line = 1 mM SNP treated Wild Type, Blue line = *katB/katG* double mutant Control, Green line = 1 mM SNP treated *katB/katG* double mutant. Analysis for RNS generation using DHR with the FITC-channel and for ROS generation using DHE with the propidium iodide channel was done. X-axis is the intensity of fluorescence in the population and Y-axis represents the bacterial count.

generator, SNP, it showed a heterogeneous response where a comparatively significant fraction (74 %) of the cell population appears to produce RNS (5 min) RNS production in cells did not change significantly following 30 min incubation in presence of SNP. Moreover, the heterogeneity in the population increased and became prominent with the ATz and SNP treatment in combination. The dual population (two populations in blue) characteristics are significantly high with the combination immediately (5 min) after treatment. However, a decent level of RNS (mean fluorescence value) was maintained in majority (67% of the high peak in blue) of the population at later time point (30 min). Only a small fraction (33 % small plateau in blue) maintained a higher level (mean fluorescence value). In Δ*katB/katG* strain (Fig. 6b), upon SNP treatment a small fraction (16 %) of cell appeared to produce significant amount of RNS with respect to its wild type (9 %) strain (5 min). After 30 min, however an increase in RNS production was observed in both SNP treated wild type (Red line) and SNP treated Δ*katB/katG* strain (Green line) as the peak was shifted towards right. Interestingly, the amount of cell fraction produced RNS in SNP treated Δ*katB/katG* strain (63 %) was much higher than that of SNP treated wild type strain of *V. cholerae* (31 %). The rightward shifting of histogram peak of SNP treated Δ*katB/katG* strain indicated that there was no heterogeneity is RNS producing cell population after 30 min, as the whole population was producing RNS. The profile of RNS production in ATz pretreated NB2 strain and Δ*katB/katG* strain was quite similar upon nitrosative stress after 5 min and after 30 min. But like NB2 strain, no generation of ROS was observed in flow Cytometry either in wild type or in Δ*katB/katG* strain even upon SNP treatment.

In cells that are capable of producing oxidation of DHR is likely to include a peroxynitrite ($ONOO^-$) component. The oxidation yield of DHR by peroxynitrite is one of the most efficient peroxynitrite-mediated oxidations determined to date [32]. Thus, it can be concluded that the fluorescence product Rhodamine generated after 5 min and 30 min following exposure to SNP in NB2, ATz pretreated NB2 and in Δ*katB/katG* strain might due to the presence of peroxynitrite species intracellularly.

3.7. Cu Zn SOD activity remained unaltered under nitrosative stress in V. cholerae

In our previous experiment we did not find any significant change in ROS generation in cell under nitrosative stress in NB2 strain of *V. cholerae* using DHE. It can be possible by two ways: either ROS is quickly scavenged by ROS detoxifying enzymes under nitrosative stress or superoxide binds with NO to produce peroxynitrite. To check that we measured Cu-Zn SOD activity of NB2 strain of *V. cholerae* cells treated separately with 50 mM ATz, 1 mM SNP and the control cells with decomposed SNP for 30 min. SOD activity was not changed significantly in NB2 strain of *V. cholerae* cells treated with 1 mM SNP (Sp. Activity 0.78 Unit/mg) for 30 min compared to the control (Sp. Activity 0.68 Unit/mg) (Fig. 7). There was a ~36% increase in SOD activity in NB2 strain of *V. cholerae* cells when the catalase activity was blocked by 50 mM ATz. To measure the status of SOD activity under catalase inhibited conditions following nitrosative stress, 50 mM ATz pretreated NB2 strain of *V. cholerae* cells were subjected to 1 mM SNP treatment. Surprisingly, SOD activity was almost similar with the 50 mM ATz treated conditions. Thus it can be concluded that SOD activity was only increased to scavenge more superoxide when the catalase activity was blocked. But when NO donor was available NO competes with SOD to bind superoxide and generates peroxynitrite.

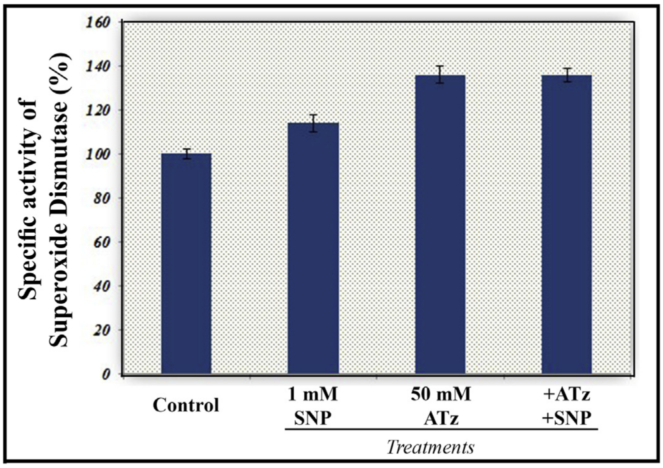

Fig. 7. Determination of Superoxide Dismutase (SOD) activity in *V. cholerae* strain NB2. SOD activity of NB2 strain was measured spectrophotometrically at 560 nm by NBT oxidation method where xanthine and xanthine oxidase was used as superoxide generator and expressed as change in percentage of specific activity as compared with control set. Specific activity of SOD was expressed in Unit/mg of protein. The calculated basal level (Control) SOD sp. activity in NB2 was 0.68 U/mg protein. This data is represented as mean ± SD (n = 3). $^{**}p < 0.01$, $^{****}p < 0.0001$.

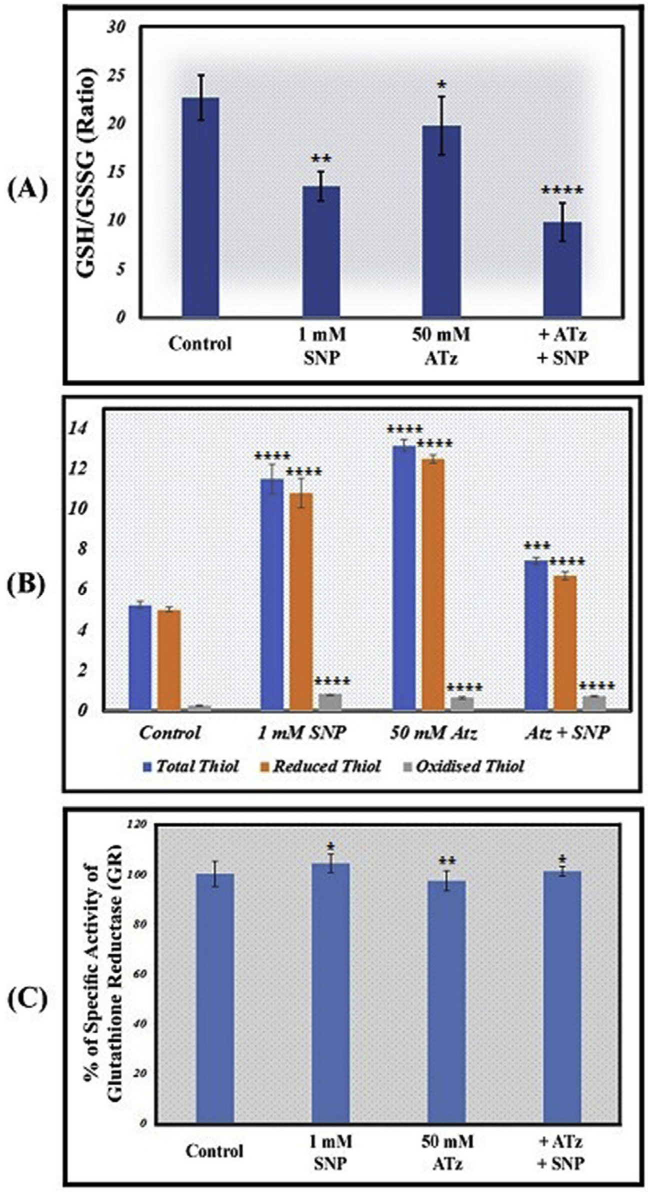

Fig. 8. Determination of Cellular redox status and activity of redox active enzymes. (A) GSH/GSSG ratio of NB2 strain of *V. cholerae*. (B) Total thiol (GSH + GSSG), Reduced thiol (GSH) and Oxidized thiol (GSSG) estimation of NB2 strain of *V. cholerae*. (C) Glutathione reductase (GR) activity of NB2 strain of *V. cholerae*. The estimated specific activities of GR were 363 nmol/min/mg protein, 378 nmol/min/mg protein, 353 nmol/min/mg protein and 365 nmol/min/mg protein in control, 1 mM SNP treated, 50 mM ATz pretreated and ATz-SNP co-treated cell lysates respectively. This data is represented as mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

3.8. Nitrosative stress alters cellular redox status in V. cholerae

Our previous study showed that reduced glutathione (GSH) play a significant role to combat nitrosative stress [15]. This fact drove us to investigate the influence of short-term nitrosative stress (30 min) on intracellular glutathione status. We measured total glutathione (GSH + 2GSSG), oxidized glutathione (GSSG), and the ratio of reduced and oxidized forms (GSH/GSSG) as markers of cellular glutathione status after treatment of NB2 strain of *V. cholerae* cells with 1 mM SNP or 50 mM ATz or 50 mM ATz pretreated cells followed by 1 mM SNP for 30 min (Fig. 8A, B, 8C). At all these above-mentioned concentrations of SNP or catalase inhibitor, cell growth was inhibited significantly in 50 mM ATz pretreated cells followed by 1 mM SNP for 30 min (~68% cell viability). Total glutathione content was increased almost 2.2 fold following 1 mM SNP treatment in NB2 strain of *V. cholerae* compared to the control. Total glutathione content was further increased almost 2.5 fold in NB2 strain of *V. cholerae* when catalase inhibitor was applied. But there was a sharp drop in total glutathione content when in 50 mM ATz pretreated cells were treated with 1 mM SNP for 30 min in NB2 strain of *V. cholerae*. However, the total glutathione content was still 1.42 fold high under nitrosative stress in the presence of catalase inhibitor in NB2 strain of *V. cholerae* compared to the control. Oxidized glutathione content was increased significantly (3.63 fold) under nitrosative stress in NB2 strain of *V. cholerae* compared to the control, the net effect of the stress was reflected in the GSH/GSSG ratio, which was decreased by 1.68 fold. The effect of nitrosative stress was more pronounced in the presence of catalase inhibitor which was 2.3 fold decrease of GSH/GSSG ratio. Thus it can be concluded that catalase plays an important role in *V. cholerae* under nitrosative stress which was reflected in the cellular redox status. We also determined the activities of glutathione reductase (GR). Specific activities of GR did not change significantly under all the above-mentioned conditions in NB2 strain of *V. cholerae*.

3.9. *V. cholerae* catalase protects against peroxynitrite-induced DHR oxidation

Several enzymes are found to be important components of cellular defense system against reactive nitrogen species (RNS) through genetic studies in bacteria and yeasts. The peroxiredoxin alkylhydroperoxide reductase subunit C (AhpC) has been shown as peroxynitrite reductase in *Mycobacterium tuberculosis*, *Salmonella typhimurium* and *Helicobacter pylori* [29,33]. Glutathione peroxidase (GPx) [34], and cytochrome *c* oxidase [35] have also been shown to protect against peroxynitrite-mediated oxidation and nitration under in vitro condition. We previously showed that in vivo activation of catalase in *Saccharomyces cerevisiae* following peroxynitrite treatment is not due to formation of reactive oxygen species (ROS) [36]. Moreover, it showed peroxynitrite reductase activity. In the present study also, the FACS analysis of DHR fluorescence supports the involvement of quenching the intracellular RNS produced upon stress (addition of the stressor or removal of the quencher) and thus indicate a novel activity of catalase. At first, we tested its ability to protect DHR 123 against peroxynitrite-mediated oxidation. At a molar excess of cell lysate over peroxynitrite, 1 mM SNP treated *V. cholerae* cell lysate protected DHR 123 oxidation up to 72% compared to the control (22%) (Fig. 9). Similar concentration of BSA could not give any protection of DHR 123 oxidation. Only 7% protection of DHR 123 oxidation was obtained when the catalase activity was blocked by the pretreatment of NB2 strain of *V. cholerae* with 50 mM ATz. Lysates prepared from NB2 strain of *V. cholerae* cells pretreated with 50 mM ATz followed by 1 mM SNP treatment could contribute almost 31% protection of DHR 123 oxidation. Thus, from the above experimental results it can be concluded that induction of catalases in *V. cholerae* cells under nitrosative stress could protect DHR123 oxidation against peroxynitrite.

4. Discussion

In spite of having several reports on the virulence factors of *V. cholerae* to cause disease cholerae, relatively little is known about the nitrosative stress response mechanisms that *V. cholerae* experience during infection of the intestine. It has been shown that *norR* actually regulates two genes *nnrS* and *hmpA* which are responsible for NO detoxification in both iNOS dependent and iNOS independent system in adult mouse model [17]. In this study we have identified another detoxification system which is specific for peroxynitrite. This is the first report where we show a novel role of *V. cholerae* catalase under nitrosative stress. We have further demonstrated that *V. cholerae* catalases are induced in the presence of sodium nitroprusside (SNP), an NO donor. Interestingly, maximum induction of catalase activity in *V. cholerae* was observed only in the presence of NO donor and not in the presence of GSNO. Catalases are essentially required for *V. cholerae* to survive under oxidative stress [22]. Catalases are also indispensable under nitrosative stress in *V. cholerae* as it is evidenced from the viable count data where blocking of catalase resulted in decreased cell survival in the presence of NO donor. ROS are produced as part of the immune response to kill the invading pathogen. NOX oxidase family enzymes are the source of ROS production in the host. At the same time intestinal epithelial cells are known to produce NO. Chemically NO radicals can scavenge the more ROS yielding peroxynitrite. NO also scavenges $GS^{\cdot}$ to produce GSNO. In our previous study we showed that GSNO reductase activity is inhibited by S-nitrosylation in *V. cholerae* under nitrosative stress. Furthermore, NO inhibits the Fenton reaction by the interaction with bioactive iron or small molecular weight iron complexes resulting in a decrease of ROS. Our flow cytometry data suggest similar effect where short exposure to NO donor did not generate ROS in *V. cholerae* cells and it supports the generation of RNS. Here NO is found to compete with SOD to produce peroxynitrite keeping SOD activity remained unchanged in *V. cholerae* under nitrosative stress. Increase in total thiol under nitrosative stress could protect *V. cholerae* even in the presence of catalase inhibitor. But decrease in GSH/GSSG ratio under nitrosative stress in the presence of catalase inhibitor also indicates that *V. cholerae* cells are facing more stress in the presence of catalase inhibitor. Peroxynitrite has a complex chemistry and addition of $ONOO^-$ leads to oxidation and/or nitration of different biomolecules by different mechanisms. GR is known for its post translational modification where active site Tyrosine is modified to 3- nitrotyrosine and thus its activity gets inhibited. Peroxynitrite could not lead to protein nitration of GR in *V. cholerae* as GR activity did not change significantly in *V. cholerae* under nitrosative stress. Peroxynitrite could not exert oxidative stress in *V. cholerae* cells as it is degraded by catalases. In our previous study we showed significantly rapid rate of reaction of catalase with peroxynitrite which suggests its ability to use peroxynitrite as a substrate and

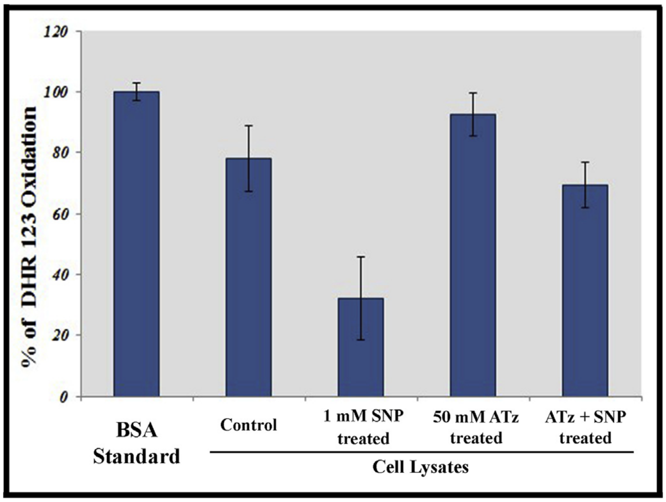

Fig. 9. Peroxynitrite mediated DHR 123 oxidation assay using SNP and ATz treated *V. cholerae* cell lysates. Results are expressed as means ± SD, (n = 5). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

thus protect cells from peroxynitrite toxicity [36]. Our report suggested that catalase can also be treated as peroxynitrite reductase with similar type of reactivity ($1.58 \times 10^6\,M^{-1}\,s^{-1}$) towards peroxynitrite and much faster than that of hemoglobin ($2.5 \times 10^4\,M^{-1}\,s^{-1}$) [37], a well-known heme protein or small non-protein molecules like glutathione ($5.8 \times 10^2\,M^{-1}\,s^{-1}$), ascorbate ($50\,M^{-1}\,s^{-1}$), or CO2 ($3 \times 10^4\,M^{-1}\,s^{-1}$) [38]. Similar result was also reported by Gebicka and Didik [39].

Vibrio cholerae keeps a low level of intracellular H_2O_2 as well as superoxide pool which has been reported [40] recently. This *in vivo* generation of ROS is governed by Na^+-NQR complex in a continuous manner. SNP as a NO donor releases nitrosonium ions (NO^+) which when enters inside cell, converted to nitric oxide radical (•NO) due to cellular nucleophilic reduction. •NO outcompetes SOD to react with superoxides due to higher rate constant [41] and forms peroxynitrite. Nitrosative stress changes the iron and copper homeostasis which also increases the Fenton reaction inside cells producing more superoxides and thus helps in formation of peroxynitrite. In our study we did not observe any significant increase in SOD activity which implied the fact that the intracellular superoxide pool might be scavenged by the NO radicals as soon as they entered into *V. cholerae* cells.

In conclusion, our reports reveal a novel role of catalase in *V. cholerae* in peroxynitrite detoxification. Catalase is well conserved from prokaryotes to higher eukaryotes. Although its oxidative stress combating properties is to remove hydrogen peroxide, still in *V. cholerae* catalase emerged as a stress protective enzyme against peroxynitrite. There must be some threshold level of peroxynitrite toxicity to induce catalase expression. Further *in vivo* works will put more insight into the involvement of transcription factor(s) responsible for such induction in gene expression.

Conflicts of interest

The authors declare no conflict of interest.

Author's contributions

SKP and NS performed the experiments. SS and SKP contributed to the Flow Cytometry analysis. PKB provided the *V. cholerae* strains. SKP and SG analyzed the results and wrote the manuscript. All authors approved the final version of the manuscript.

The authors declare no financial or other conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.04.002.

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