**INTRODUCTION**

*Vibrio cholerae* pathogenesis is multi-factorial and relies on both host and bacterial counterparts. *V. cholerae* after being introduced into the intestinal lumen requires attachment to intestinal linings. This initial attachment is the key step in pathogenesis of *V. cholerae*. *N*-Acetylglucosamine-binding protein A (GbpA), the gene product of locus VCA0811, serves an important role as a common adhesin for chitinous surfaces as well as intestinal epithelium (Kirk *et al.*, 2005; Bhowmick *et al.*, 2008; Wong *et al.*, 2012). It was identified as an adhesion factor for surfaces with *N*-acetylglucosamine (GlcNAc) and its oligomers (Meibom *et al.*, 2004). In spite of being secretory in nature, GbpA has been found to be the factor bridging *V. cholerae* and mucin in the host (Bhowmick *et al.*, 2008). The GbpA was found to increase the production of intestinal secretory mucins (MUC2, MUC3 and MUC5AC) in HT-29 cells through the activation of corresponding genes (Bhowmick *et al.*, 2008). The activation of mucin genes (MUC2 and MUC5AC) was found to be dependent on nuclear translocation of NF-κB (Bhowmick *et al.*, 2008). Therefore, GbpA can be considered as an effector molecule capable of eliciting host response.

Vibrio cholerae N-acetylglucosamine-binding protein GbpA is a secretory protein that facilitates the initial adherence of bacteria in the human intestine. Until now, considerable progress in the characterization of GbpA has been done, yet little is known about its role in host response. Our present studies demonstrated that GbpA at the amount secreted in the intestine resulted in decreased cell viability, altered cell morphology, disruption of cell membrane integrity and damage of cellular DNA indicating necrotic cell death. We observed that GbpA exposure leads to mitochondrial dysfunction, characterized by accumulation of reactive oxygen species (ROS), depolarization of mitochondrial membrane potential and depletion of ATP pool in host cells. Additionally, the intracellular ROS, accumulated in response to GbpA, were found to induce the migration of NF-κB from cytoplasm into nucleus in host cells. Taken together, these results prompted us to conclude that GbpA orchestrates a necrotic response in host cells which may have implications in immune response.

**Vibrio cholerae** GbpA elicits necrotic cell death in intestinal cells

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Vibrio cholerae *N*-acetylglucosamine-binding protein GbpA is a secretory protein that facilitates the initial adherence of bacteria in the human intestine. Until now, considerable progress in the characterization of GbpA has been done, yet little is known about its role in host response. Our present studies demonstrated that GbpA at the amount secreted in the intestine resulted in decreased cell viability, altered cell morphology, disruption of cell membrane integrity and damage of cellular DNA indicating necrotic cell death. We observed that GbpA exposure leads to mitochondrial dysfunction, characterized by accumulation of reactive oxygen species (ROS), depolarization of mitochondrial membrane potential and depletion of ATP pool in host cells. Additionally, the intra-cellular ROS, accumulated in response to GbpA, were found to induce the migration of NF-κB from cytoplasm into nucleus in host cells. Taken together, these results prompted us to conclude that GbpA orchestrates a necrotic response in host cells which may have implications in immune response.

Necrosis is a type of inflammatory cell death characterized by membrane rupture, nuclear disruption and the concomitant release of cellular contents. Necrotically dying cells can initiate pro-inflammatory signalling cascades by effectively releasing inflammatory cytokines and by spilling their cellular contents when they rupture. Reactive oxygen species (ROS) have been documented to be a major factor in the propagation of necrotic cell death (Festjens *et al.*, 2006). Necrosis can also be triggered by danger signals like externalization of phosphatidylserine (PS) on cell membrane, depletion of ATP molecules, mitochondrial dysfunction, etc., which can be induced upon bacterial infection (Golstein *et al.*, 2007). Host cell response including release of pro-inflammatory cytokines and cell death due to ROS generation are well described in the case of invasive *Vibrio vulnificus* infection (*Lee et al.*, 2015a, b). *V. cholerae* cytolsin was found to induce autophagy, vacuolation and apoptosis (*Coelho et al.*, 2000; Gutierrez *et al.*, 2007; Saka *et al.*, 2008) as well as secretion of pro-inflammatory cytokine IL-8 (*Ou et al.*, 2009) in cell culture models. However, there is a dearth of instances of ROS accumulation and cell necrosis in *V. cholerae* infection owing to the facts that *V. cholerae* is non-invasive in nature and cholera pathogenesis is unrelated to intestinal inflammation.

Until now, considerable progress in the characterization of GbpA has been made, yet little is known about its role in host response. In this current study, we investigated the role of *V. cholerae* GbpA in eliciting host response in HT-29 gastrointestinal cells. We report that GbpA treatment elicits host response in the form of cell necrosis, ROS generation and activation of NF-κB in tissue culture models (HT-29 cells).

**Abbreviations:** DCFDA, 2',7'-dichlorofluorescein diacetate; FACS, fluorescence-activated cell sorting; FSC, forward scattering; LDH, lactate dehydrogenase; MMP, mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species; PI, propidium iodide; PS, phosphatidylserine; SSC, side scattering; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling.
METHODS

Bacterial strain and protein preparation. *V. cholerae* O1 El Tor strain N16961 was used to determine the amount of GbpA secreted in infected rabbit intestine. The bacteria were cultured in Luria–Bertani medium at 37°C. *V. cholerae* GbpA protein was expressed in BL21 Star (DE3) cells harbouring the pET22b vector encoding the gbpA gene (VCA0811), as described previously (Wong et al., 2012).

Cell culture. Human intestinal cell line HT-29 was procured from NCCS. The cells were routinely maintained in DMEM (Dulbecco's Modified Eagle Medium) (Sigma) supplemented with 10% foetal bovine serum, 100 U of penicillin ml⁻¹ and 100 µg of streptomycin ml⁻¹ (MP Biomedicals) in 5% CO₂ environment at 37°C.

Determination of amount of GbpA secreted in the rabbit intestine. The standard curve of GbpA was prepared by indirect ELISA method using 0.01–2 µg ml⁻¹ of purified GbpA and 1: 500 anti-GbpA antibody raised in mouse. The standard curve regression equation was generated from the curve. The amount of GbpA secreted from *V. cholerae* in adult rabbit intestine was determined using the ileal loop method, as reported previously (De et al., 2015; 10³ c.f.u. of *V. cholerae* N16961 in 1 ml PBS was inoculated in a 10 cm loop. Fluid accumulated within the inoculated loop was collected for quantification of GbpA. The concentration of GbpA present in accumulated fluid was determined using the standard curve equation of GbpA.

Assessment of morphological changes. HT-29 cells treated with (250 ng µl⁻¹) or without GbpA were incubated for 24h. The morphological alterations in the GbpA-treated cells were observed under phase contrast microscope using ×40 objective (Zeiss). Cells were also imaged by fluorescence microscope (Zeiss) after immunostaining of actin cytoskeleton using anti-actin primary antibody and AlexaFluor 488-conjugated secondary antibody followed by counterstaining with 4', 6-diamidino-2-phenylindole (DAPI). Alterations in cell size and complexity in GbpA-treated cells were examined by flow cytometer under the parameters applicable for forward scattering (FSC) and side scattering (SSC), as described previously (Gupta et al., 2015). Here, FSC correlates with the measure of cell size and shape, whereas SSC indicates relative cell granularity, refractivity and presence of intra-cellular structures that can reflect the light. For this study, cells treated with or without GbpA were re-suspended in the staining buffer containing 0.1 mg ml⁻¹ propidium iodide (PI) in 0.6% Triton-X in PBS and washed twice with PBS before fluorescence-activated cell sorting (FACS) analysis.

MTT-cell viability assay. In this assay, 5 x 10⁴ HT-29 cells well⁻¹ were seeded in 96-well plates and treated with increasing concentration of GbpA for 24h. Untreated cells were kept as control. Cell viability was determined by the 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-based cell growth determination kit (Promokine) as per recommended protocol.

Lactate dehydrogenase release assay. HT-29 cells (5 x 10⁴ well⁻¹) were seeded in a 96-well plate and treated with GbpA according to the protocol mentioned in MTT assay. The release of lactate dehydrogenase (LDH) in the culture supernatant was monitored by colorimetric method using the LDH Cytotoxicity assay kit (Takara Bio) following the manufacturer’s protocol.

FACS-based annexin V-FITC/PI double staining assay. GbpA-treated HT-29 cells were double stained using annexin V-FITC/PI (BD Biosciences), and the percentage of cell population undergoing flipping of PS was evaluated using flow cytometer. Untreated cells were kept as control. For flow cytometer analysis, data acquisition was conducted on a FACS Aria cytometer using a FACS Diva data management system (BD Biosciences).

DNA ladder assay. The effect of GbpA treatment on nuclear DNA of HT-29 cells was detected by DNA ladder assay using the DNA ladder isolation kit (Abcam). Cells treated with H₂O₂ were taken as positive control for cell necrosis. Untreated cells were set up as negative control.

TUNEL staining. The physical status of the cell nucleus upon GbpA treatment was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining using the APO Direct kit (BD Biosciences). After treatment for 24h, cells were subjected to dual staining with TUNEL and PI as stated in the kit protocol. TUNEL-stained cells were monitored by fluorescence microscopy under oil immersion objective.

Detection of ROS and mito-ROS. To determine total intra-cellular ROS and mito-ROS, fluorescence microscopy and flow cytometer analysis were carried out. Cells were treated with GbpA (250 ng µl⁻¹) and incubated for 8h at 37°C. The cells were washed with PBS and incubated with 2',7'-dichlorofluorescein diacetate (H₂DCFDA; Abcam) at a final concentration of 40 µM. For flow cytometer examination, a FACS Canto II cytometer (BD Biosciences) was used. Cells treated with GbpA in presence of N-acetyl-L-cysteine (NAC) were set up as experimental control to determine the specificity of GbpA in provoking ROS generation in host cells. MitoSOX (Invitrogen) was used to detect mitochondrial ROS at a final concentration of 10 µM in serum-deficient DMEM in GbpA-treated cells incubated for 30min at 37°C. Apocynin

Fig. 1. (a) Standard curve of GbpA. Different concentrations of GbpA, i.e. 0.01, 0.02, 0.05, 0.1, 0.5, 1, 1.5 and 2 µg µl⁻¹, were coated in the wells of microtitre plate, and amount of colour developed was quantified using polyclonal anti-GbpA raised in mouse at a dilution of 1:500 by measuring the absorbance at 450 nm. (b) GbpA induces loss in cell viability. Estimation of viability of HT-29 cells upon treatment with increasing concentration of GbpA for 24 h was monitored by the MTT-based assay. The bar diagram represents the mean±SE of three independent measurements.
(Santa Cruz), an inhibitor of non-mitochondrial ROS generation, was used to confirm mitochondria as the major source ROS in target cells. For fluorescence microscopy study, the cells were mounted with Pro-Long Gold Anti-fade Reagent with DAPI (Invitrogen), and images were recorded using a confocal microscope (LSM710; Carl Zeiss) at ×63 magnification. To directly monitor the ROS production in live cells using fluorescence microscopy, a Cellular ROS Detection Assay kit was used (Abcam). NAC was used to inhibit ROS production wherever applicable. All data shown are illustrative of three independent experiments.

**Fig. 2.** GbpA induces morphological distortions in HT-29 cells. (a) Alteration in cell morphology upon GbpA treatment was monitored under phase contrast microscope. The black arrows indicate the damaged cells in treatment group. Bar, 10 µm. (b) Organization of the cytoskeleton in HT-29 cells with or without GbpA treatment was monitored by immunostaining. The cytoskeleton and nuclei were stained with FITC-conjugated antibody and DAPI, respectively. Bar, 20 µm. (c) Cell size and granularity in GbpA-treated or untreated cells were detected by FACS analysis. Decrease in FSC and concomitant increase in SSC in cell population compared to untreated control group indicate substantial alteration in cell morphology.

**Nuclear and cytoplasmic protein extraction and immunoblot analysis.** The HT-29 cells were ruptured in equal volumes of ice-cold lysis buffer containing protease inhibitor cocktail. Nuclear and cytoplasmic extracts were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to manufacturer’s provided protocol. The extracts were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. In each case, 30 µg of total protein was loaded in each well. After probing with specific primary (Cell Signaling Technology) and secondary antibodies (Sigma), membranes were developed with ECL system (Millipore).
Immunofluorescence assay to detect the nuclear translocation of NF-κB. Samples were prepared as described previously (Yuan et al., 2013) with little modifications. Briefly, cells were seeded on the coverslips and grown overnight and treated with GbpA (250 ng µl⁻¹) for 45 min. Cells were then incubated with primary antibodies against NF-κB-p65 (Cell Signaling Technology) diluted in PBS (1:400) for overnight at 4 °C. Cells were washed thrice in PBS and incubated with Alexa Fluor 488-conjugated secondary antibody (Abcam) for 1 h. The cells were mounted with ProLong Gold Anti-fade Reagent having DAPI (Invitrogen) as a counterstain. Images were acquired in a confocal microscope (LSM 710; Carl Zeiss) at 63× magnification. MG-132 (1 µM) served as inhibitor of NF-κB activation, and the cells were processed identically following GbpA treatment. Cells pre-treated with NAC were also stained for imaging purposes.

Determination of ATP level. ATP level in GbpA-treated HT-29 cells was measured by the bioluminescent ATP measurement kit (Sigma) following the manufacturer’s protocol. The light emitted was quantified using a luminometer (Berthelot). The level of ATP is represented as nmol of ATP (mg protein)⁻¹.

Detection of mitochondrial membrane potential transition. The depolarization of mitochondrial membrane potential (MMP) in GbpA-treated cells was investigated by the Mitotracker Red CMXros and JC-1 fluorescent probes. The cells treated with or without GbpA were stained with Mitotracker Red (Invitrogen) and imaged under fluorescence microscope under ×100 objective. Valinomycin was applied as control reagent to induce dissipated membrane potential. The mean fluorescence intensity was measured in each case by using the ImageJ software. Cells treated with (250 ng µl⁻¹) or without GbpA were stained with JC-1 (BD Biosciences) and were subjected to fluorescence microscopy. The data are represented as the ratio of red to green fluorescence intensity. A decrease in the ratio of red/green in GbpA-treated cells compared to that of untreated cells is considered a reduction in MMP (Δψm).

Statistical analysis. Most of the experiments were executed in triplicate. Data are represented as means±SE. The data are analysed by one-way ANOVA applying GraphPad Prism version 5.0 for Windows. P<0.05 was considered statistically significant.

RESULTS

V. cholerae secretes a significant amount of GbpA in the rabbit intestine

The regression equation obtained from the standard curve of GbpA was used to calculate the amount of GbpA secreted in the rabbit intestine (Fig. 1a). The concentration of GbpA was found to be 330 ng µl⁻¹ in the rabbit ileal loop assay.
GbpA treatment induces dose-dependent death of intestinal cells

HT-29 cells were treated with different concentrations of GbpA for 24 h, and cell viability was examined using MTT assay. The result showed that the cells lost viability with increasing doses of GbpA (Fig. 1b). In response to 35 ng µl⁻¹ of GbpA, 25 % loss of cell viability was observed. Loss in cell viability was 50 and 75 % with treatments of 250 and 500 ng µl⁻¹ of GbpA, respectively. untreated cells were 100 % viable. These observations suggested that GbpA treatment impaired target cell health and induced loss in cell viability.

GbpA induces morphological distortions in host cells that indicate necrosis

The effect of GbpA on the morphology of HT-29 cells was examined. Rounding of cells, poor adherence and appearance of cell debris revealed the necrotizing effect of GbpA on intestinal cells. Untreated cells were intact in cell morphology (Fig. 2a). Alteration in the arrangement of actin filaments in HT-29 cells treated with GbpA was visualized by fluorescence staining of cytoskeleton. The untreated cells were rich in actin filaments with regular parallel organization, and cell morphology appeared intact. Treatment with GbpA for 24 h caused perturbation of cytoskeletal organization. The actin filaments became disordered and chaotic in appearance and the cell morphology indicated features of cell necrosis (Fig. 2b).

The changes in cell morphology, observed under phase and fluorescence microscopy, were further confirmed by flow cytometry as dot plots of dual parameters FSC vs SSC (cell size vs granularity, density). Comparative analysis by bi-parametric system (dot plot) of FSC vs SSC for cells treated with GbpA showed a distinct shift of the main cell population towards the Y-axis (i.e. SSC) (Fig. 2c). This implied a decrease in FSC and an increase in SSC. This suggested that GbpA induces shrinkage of cell size and shape (FSC) along with increased relative cell granularity (SSC) in target cells, which was found quite consistent with the earlier imaging results.

GbpA treatment leads to externalization of PS in target cell membrane

The effect of GbpA treatment on the biochemical changes in the host cell membrane was evaluated by the detection of externalized PS on the outer leaflet of cell membrane. To examine GbpA-induced flipping of PS in target cell membrane, the HT-29 cells were treated with different doses of GbpA and incubated for 24 h. In the flow cytometry experiment, GbpA-treated cells were annexin V/PI double positive. This population increased in a dose-dependent manner from 125 to 1000 ng µl⁻¹ of GbpA (Fig. 3). Treatment with 125 ng µl⁻¹ of GbpA gave rise to 28 % late apoptotic cells. Treatment with 1000 ng µl⁻¹ of GbpA gave rise to 60 % late apoptotic cells.

Fig. 4. GbpA-induced DNA damage in HT-29 cells. (a) Analysis of DNA integrity in GbpA-treated HT-29 cells by agarose gel electrophoresis. HT-29 cells were incubated with GbpA for 8 and 24 h. DNA from the cells was extracted and electrophoresed on a 2 % agarose gel and visualized using a UV trans-illuminator. H₂O₂ (0.8 mM) treated and untreated lanes represent positive and negative controls, respectively. (b) Dual staining with PI and TUNEL showed the presence of fragmented DNA molecules in the GbpA-treated cell population. The stained nucleus of both treated and untreated cells was imaged under fluorescence microscope. Bar, 20 µm.

Fig. 5. GbpA treatment causes increased cell membrane permeability. Cell membrane disrupting activity of GbpA in HT-29 cells was detected by LDH release assay. The data presented are the mean±SE of three independent measurements.
**GbPA induces DNA damage in target cells typical of necrotic cells**

The integrity of DNA of target cells following GbPA treatment was tested by DNA ladder assay and TUNEL staining. The result showed a significant reduction in the number of TUNEL-positive cells compared to the control. The degradation of DNA was observed in the form of distinct DNA ladder formation, indicating apoptosis. The TUNEL assay confirmed the apoptosis in GbPA-treated cells, as evidenced by the increased fluorescence intensity in the nuclei, indicative of DNA fragmentation.(Fig. 4a).

**GbPA induces increased cell membrane permeability**

Next, LDH release assay was done to analyse cell membrane leakage during GbPA treatment. Different doses of GbPA were used to treat HT-29 cells for 24 h and the supernatants were collected. Supernatants were assessed for LDH release. The result showed a significant increase in LDH release from GbPA-treated cells compared to untreated cells (Fig. 5). The above results led to the conclusion that GbPA-mediated cell death could have characteristic of necrosis.

**GbPA elicits mitochondrial ROS accumulation in host cells**

Since the results obtained so far were indicative of necrotic cell death, accumulation of intracellular ROS in host cells was measured. Results showed that GbPA significantly increased ROS levels compared to untreated cells, indicating mitochondrial dysfunction. The fluorescence microscopy results showed that GbPA increased ROS and superoxide production in host cells compared to untreated cells. (Fig. 6a).

Next, the HT-29 cells were stained with MitoSOX dye and with Z (DCFDA) to detect the cellular ROS concomitantly. The confocal images showed red fluorescence from mito-ROS co-localized with the green fluorescence of DCF-Ros in GbPA-treated cells. In the case of untreated cells, only background fluorescence of DCF was detected. NAC, an antioxidant, significantly reduced the ROS and superoxide accumulation in the cells (Fig. 6b).

**GbPA induces mitochondrial dysfunction in target cells**

In order to understand whether the GbPA-mediated cell death mechanism involved mitochondrial dysfunction, HT-29 cells were treated with different doses of GbPA. Treated cells were examined for alteration in MMP (Δψm), using MitoTracker Red CMXros dye. The result showed a decrease in Δψm, indicating mitochondrial membrane depolarization. The depolarization of MMP was further confirmed by staining the GbPA-treated cells with JC-1 dye. The fluorescence images indicated that the red J-aggregates converted into green J-monomers upon stimulation with GbPA. (Fig. 7a, b).

Mitochondrial dysfunction often leads to decrease in cellular ATP levels. The cellular content of ATP following GbPA treatment was estimated. The result suggested that GbPA treatment caused a twofold reduction in ATP level compared to the untreated cells (Fig. 7d).

**GbPA induces nuclear translocation of NF-κB-p65 in host cells**

Following GbPA treatment, activation of NF-κB in response to GbPA was evaluated. The confocal images of HT-29 cells stained for NF-κB-p65 indicated that the protein was mainly sequestered in the cytosol in untreated cells. Treatment of cells with GbPA led to the migration of NF-κB-p65 from cytosol to nucleus within 45 min post-stimulation. The NF-κB inhibitor MG-132, blocked the activation of GbPA-induced NF-κB activation (Fig. 8a).

**Influence of ROS on NF-κB activation in GbPA-treated cells**

Next, we looked into whether the intra-cellular ROS accumulated in response to GbPA could influence the nuclear translocation of NF-κB in target cells. The effect of intracellular ROS in the translocation of NF-κB-p65 was investigated by neutralizing the ROS with antioxidant NAC. The confocal images indicated that pre-treatment of cells with NAC for 1 h blocked the migration of NF-κB-p65 from cytosol to nucleus (Fig. 8b). The immunoblots of cytosolic and nuclear extracts from HT-29 cells stimulated with GbPA further confirmed the successive changes in localization of the NF-κB-p65 protein. Here, it was also found that pre-treatment of cells with NAC inhibited the translocation process (Fig. 8c).
DISCUSSION

Results presented here showed that GbpA can induce necrotic cell death in intestinal cells. Different bacterial proteins can induce a number of host cellular responses, and cell death is one of them. A study on *V. cholerae* porin OmpU suggested that it has the ability to induce target cell death (Gupta et al., 2015). Recently, a group reported that outer membrane vesicle associated biologically active proteases from *V. cholerae* can induce cytotoxic and inflammatory response in host cells (Mondal et al., 2016). A recent report on *V. cholerae* haemagglutinin protease suggested effective apoptosis of breast cancer cells (Ray et al., 2016). These emerging reports on targeted cell death by *V. cholerae* proteins suggest that our observation of cell death induced by GbpA is also possible.

Further insights into the mechanism of cell death induced by GbpA revealed characteristics of necrosis. In healthy cells, PS molecules are distributed asymmetrically on the cell membrane and majorly found in abundance in the inner cell membrane. In cells undergoing necrosis, PS externalization takes place owing to membrane blebbing, and its availability increases in the outer leaflet of the cell membrane (Zao et al., 2015). Our data also indicated that GbpA induces externalization of PS in intestinal cells.

Increase in cell membrane permeability indicates compromised cell membrane integrity. This leads to the spillage of cellular contents in the extra-cellular milieu which might provoke inflammatory reactions in neighbouring cells (Matzinger, 1994; Shi et al., 2000). Here, presence of GbpA was found to increase cell membrane permeability in host cells which is indicative of cell necrosis.

The integrity of cellular DNA reflects the physical status of host cells. In apoptotic cells, DNA fragmentation takes place which appears as a DNA ladder in ladder assay (Ioannou &

![Image](http://jmm.microbiologyresearch.org)
Chen, 1996; Suman et al., 2012). On the contrary, in necrotic cells, DNA is cleaved into fragments of random size by non-specific lysosomal nucleases that generate a DNA smear on agarose gel electrophoresis. Here, the occurrence of smearing pattern of DNA isolated from the GbpA-treated cells indicates cell necrosis. Appearance of TUNEL-positive cells further strengthened our hypothesis of GbpA-induced necrotic cell death. Bacterial components have been found to trigger host cell death with some features of necrosis (Lamkanfi et al., 2010).

GbpA exposure could also elicit accumulation of intracellular ROS in host cells. An earlier study showed that V. cholerae infection stimulates ROS production in human neutrophils (Seper et al., 2013). Furthermore, the mitochondria of cells were the main contributor of ROS generation. GbpA-mediated ROS generation suggested that it could induce mitochondrial dysfunction and depletion of ATP pool. Depolarization of MMP usually affects the electron transport chain which may result in the generation of ROS and depletion of ATP pool in affected cells (Kirkinezos et al., 2001; Tranciková et al., 2004). Our results also indicated ATP depletion in GbpA-treated cells.

Previous reports have shown a correlation between ROS production and functional deterioration and progression in necrotic cell death (Morgan et al., 2008; Simon et al., 2009; Choi et al., 2009). Similar types of events was also observed in this study. Thus, it can be extrapolated that the ROS generation following GbpA exposure could contribute to the cell necrosis.

Another significant finding of our study was the activation of transcription factor NF-κB. Previously, it was demonstrated that the induction of mucin genes (Bhowmick et al., 2008) and secretion of pro-inflammatory cytokine IL-8 (Chatterjee et al., 2012) in intestinal cells by GbpA were regulated by NF-κB. Further influence of ROS on NF-κB

**Fig. 7.** GbpA causes mitochondrial dysfunction in HT-29 cells. (a) Confocal images of HT-29 cells treated with or without GbpA for 24 h and then loaded with Mitotracker Red. Bar, 10 μm. (b) Mean fluorescence intensities of Mitotracker Red CMXros from each treatment group were plotted. (c) Localization of JC-1 in HT-29 cells treated with GbpA (250 ng μl⁻¹) or without (PBS) was monitored by confocal microscopy. Green fluorescence under blue excitation corresponds to monomer fluorescence, and red fluorescence under green excitation corresponds to J-aggregates fluorescence. The data are represented as red to green fluorescence ratio. (d) Cellular ATP content in GbpA-treated (250 ng μl⁻¹) and untreated cells was evaluated by bioluminescent ATP measurement technique. The data were normalized in each case by equal amount of protein. The error bars indicate average±SE of three independent measurements and the asterisks signifies *P<0.05.
activation induced by various environmental stimuli is reported. However, the relationship of ROS and NF-κB activation pathway has been found to be quite complex and multi-faceted (Lang et al., 2000; Montiel-Duarte et al., 2004; Saile et al., 2001). NF-κB-inducing kinase and IKKe/IKKβ are reported as key kinases and act in a cascade to activate the NF-κB pathway (Tang et al., 2004). Study revealed that increased endogenous ROS facilitates the activation of NF-κB-inducing kinase and subsequent phosphorylation of IKKe and IKKβ as well as degradation of IKKe which, in turn, results in the activation of NF-κB (Wang et al., 2007). Studies have also delineated the signalling pathways leading to NF-κB activation by H₂O₂ in HeLa cells (Storz et al., 2003, 2004) where H₂O₂ induces IKKβ activation and NF-κB transcriptional activity via activation of protein kinase D. Here, the activation of NF-κB by GbpA was found to be dependent on the ROS, since ROS scavenger NAC effectively inhibited the process. Therefore, it can be assumed that the similar signalling events in intestinal epithelial cells might occur to accomplish the NF-κB activation in presence of GbpA through the involvement of endogenous ROS.

Collectively, it can be said that GbpA is an active bacterial component eliciting host response in the form of necrotic cell death characterized by cellular DNA damage, PS flipping, leaky cell membrane, cell rounding and increased cell granularity. Moreover, GbpA was found to induce mitochondrial dysfunction characterized by decrease in MMP, depletion in ATP content and accumulation of intra-cellular ROS in intestinal cells. Additionally, it was demonstrated that GbpA-induced activation of NF-κB was closely linked with the ROS generation.

*V. cholerae* is non-invasive, and necrotic cells are rarely visible in biopsy samples from cholera patients. Therefore, any possible intestinal tissue damage due to necrosis may be replenished readily beyond its detection, since cholera is a self-limiting disease. Another possible explanation for non-availability of necrotic cells after bacterial infection may be that the necrotic cell corpses are readily engulfed by the sensitized surveillance cells at the site of infection. Further studies are required to understand the intestinal tissue damage during *V. cholerae* pathogenesis.

Sequencing analysis of the 488 *V. cholerae* strains, originating from different cholera-affected regions in the world, suggests that the *gbpA* gene is highly conserved within the species (Stauder et al., 2012). Interestingly, the gene is also present in environmental strains belonging to other *Vibrio* species (Stauder et al., 2012). Interestingly, the gene is also present in environmental strains belonging to other *Vibrio* species.
spp., including *Vibrio alginolyticus*, *Vibrio metshchikovii*, *Vibrio mimicus*, *V. vulnificus* and *Vibrio parahaemolyticus*. Recent report suggested that, in *V. vulnificus*, the mucin-binding protein GbpA is essential for pathogenesis (Kyung et al., 2016). BLAST analysis with *V. vulnificus* GbpA revealed 76% sequence similarity in protein level with that of *V. cholerae*. Additionally, GbpA-like proteins are present in several other pathogenic, non-*Vibrio* bacteria, such as *Listeria monocytogenes*, *Bacillus cereus* and *Yersinia pestis* (Loose et al., 2014). Therefore, it can be speculated that the GbpA and GbpA-like proteins are ubiquitous in *Vibrio* as well as in other pathogenic organisms and likely to function in a similar way.

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