ROS mediated antibacterial activity of photoilluminated riboflavin: A photodynamic mechanism against nosocomial infections

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ABSTRACT

Nosocomial infections are a major threat to modern therapeutics. The major causative agent of these infections is multidrug-resistant gram-negative bacteria, which impart high morbidity and mortality rate. This has led to an urge for the development of new antibiotics. Antimicrobial photodynamic therapy is a promising strategy to which till date no resistant strain has been reported. Since the efficacy of photodynamic therapy largely depends on the selection and administration of an appropriate photosensitizer, therefore, the realization of clinically active photosensitizers is an immediate need. Here, by using E. coli as a study model we have demonstrated the antimicrobial photodynamic potential of riboflavin. Intracellular ROS formation by DCFH-DA assay, lipid peroxidation, protein carbonylation, LDH activity was measured in treated bacterial samples. Enzymatic (SOD, CAT, GSH) antioxidants and non-enzymatic (GSH) was further evaluated. Bacterial death was confirmed by colony forming assay, optical microscopy and scanning electron microscopy. The treated bacterial cells exhibited abundant ROS generation and marked increment in the level of oxidative stress markers as well as significant reduction in LDH activity. Marked reduction in colony forming units was also observed. Optical microscopic and SEM images further confirmed the bacterial death. Thus, we can say that photilluminated riboflavin renders the redox status of bacterial cells into a compromised state leading to significant membrane damage ultimately causing bacterial death. This study aims to add one more therapeutic dimension to photilluminated riboflavin as it can be effectively employed in targeting bacterial biofilms occurring on hospital wares causing several serious medical conditions.

1. Introduction

Nosocomial infections are an enormous health hazard with an estimated frequency of 1 in every 10 patients [1]. Although around one-third of nosocomial infections are preventable, still they pose a major threat to patient’s health so much so that these infections were marked as the sixth leading cause of death in the United States [2]. The increasing magnitude of these infections owes to the emergence of multidrug-resistant bacteria exhibiting resistance to a broad range of antibacterial chemotherapeutic drugs [3]. This emerging medical and social catastrophe has prompted the development of alternative pathogen inactivation techniques, hence, marking the closure of the “antibiotic era” [4].

Reportedly, gram-negative bacteria account for more than 30% ofnosocomial infections predominantly causing ventilator-associated pneumonia and urinary tract infections [5]. More so, infections arising in intensive care units (ICUs) have been predominantly caused by gram-negative bacteria [6]. E. coli is the most rampant nosocomial gram-negative pathogen causing many health issues [7]. It is a common colonizer of many medical devices [8] which makes it one of the prominent pathogen of this global health concern. E. coli thus serves as a model for determining alternative antibacterial strategies.

Among several antibacterial strategies including UV radiation, hydrogen peroxide, chlorination, and photodynamic inactivation, only photodynamic inactivation or photodynamic therapy (PDT) is relatively milder and human-friendly. It has emerged as an excellent non-invasive therapeutic modality against various bacterial infections [9]. PDT involves generation of reactive oxygen species by a photosensitive compound upon photolllumination. These reactive oxygen species then drive cell towards its death [10]. The photodynamic killing is primarily dependant on the efficacy of the applied photosensitizer and its chemically inert behavior in absence of light. Antimicrobial photodynamic
therapy is, therefore, best possible answer to this growing global health concern, as till date, no reports have shown the existence of a PDT resistant bacterial strain [11].

Riboflavin or vitamin B₅ is an essential micronutrient, which exhibits excellent photosensitive characteristics [12]. Its usage in developing pathogen inactivation technology has shown significant results [13]. In this study, we are showing the significant photodestruction of E. coli in presence of photoilluminated riboflavin. We have carried out this work in anticipation of the development of vitamin-based phototherapy against E. coli born nosocomial infections.

2. Material and methods

2.1. Bacterial strains and growth conditions

Riboflavin photosensitivity was evaluated against E. coli provided by the Department of Microbiology, AMU. Bacteria were harvested from their colonies by centrifugation and their suspensions were made using phosphate buffer saline (pH 7.4) with a final concentration of 10⁷ CFU/ml. The isolated E. coli strains were tested against cefepime, ceftazidime, cefotaxime, ceftriaxone, ceftizoxime, cefepoxime, levofloxacin, ofloxacin, and amikacin using the classical disc diffusion method.

2.2. Irradiation procedure

All the reactions were performed by irradiating samples with visible white light [Philips, India] kept at 10 cm. Irradiation rate at this point was 38.6 W m⁻² as measured by a power meter (model: Laser Mate Coherent).

2.3. Photosensitizer

Riboflavin was purchased from Sigma Aldrich (India). 50 μM riboflavin solution was taken as a working solution. UV spectra of riboflavin were recorded on Shimadzu dual beam UV spectrophotometer UV-1800 (Japan) before and after 2 h. of photoillumination.

2.4. Detection of superoxide radical in solution

Reduced nitroblue tetrazolium (NBT) method [14] was applied to measure superoxide generation potential of riboflavin. 50 μM riboflavin was added to the reaction mixture containing 33 mM NBT, 100 μM EDTA and 50 mM sodium phosphate buffer at pH 8.0 and 0.06% Triton X 100 and the mixture was read at 560 nm.

2.5. Preparation of samples

Bacterial cell suspensions were treated with 50 μM riboflavin and incubated for 2 h. in presence of light; 50 μM riboflavin in absence of light. Light and dark controls of E. coli were also maintained.

2.6. Detection of ROS in bacterial cell

The intracellular ROS was estimated using DCFH-DA method [15,16]. The treated bacterial cell suspensions were centrifuged at 4 °C for 30 min at 300 g and the supernatant was treated with 100 μM DCFH-DA for 1 h. The fluorescence intensity was recorded using Shimadzu RF5301PC spectrofluorophotometer (Japan) with an excitation and emission wavelength at 485 nm and 530 nm.

2.7. Preparation of lysates of treated bacterial cells

The treated bacterial cell suspensions were centrifuged at 4 °C for 10 min at 5000 rpm. The pellet was collected and resuspended in bacterial lysis buffer, lysozyme was added, and the sample was incubated at 4 °C. After 4 h. of incubation cells were centrifugated at 10,000 rpm, the supernatant was collected to carry out antioxidant enzyme assays.

2.8. Total protein estimation

Total protein level in the bacterial lysate was determined using the Lowry Method [17].

2.9. Measurement of SOD enzyme activity

The cell lysate (prepared as described above) of the treated bacterial cell suspensions was taken to evaluate the activity of SOD [18,19].

2.10. Measurement of catalase activity

To determine catalase activity 100 μl bacterial lysate was mixed with a reaction mixture containing 0.1 M phosphate buffer (pH 7.5) and 0.9% H₂O₂ solution in a ratio 1:1 (500 μM) and incubated for 3 min. 2N H₂SO₄ was added at the end of the experiment to stop the reaction and the activity was calculated as described before [15,19].

2.11. Measurement of total GST activity and GSH level

Total GST activity (μmol/min/mg protein) was evaluated as described before [20]. 25 μl of sample is added to the reaction mixture containing 900 ml of 100 mM potassium phosphate buffer (pH 6.5), 25 ml of 40 mM CDNB and 50 ml 1 mM GSH. The increase in absorbance was recorded at 340 nm for a period of 5 min. GSH level was estimated by the DTNB method as described before [21]. The absorbance was read at 412 nm.

2.12. Measurement of lipid peroxidation

The amount of lipid peroxidation in bacterial suspensions was determined by assaying thiobarbituric acid-reactive substances formed [22–24]. 10% SDS was added in treated cell samples and mixed thoroughly. After that, freshly prepared TBA was added to them and samples were incubated at 95 °C for 60 min. Finally, samples were centrifuged at 5000 rpm for 15 min., the supernatant was collected to determine OD at 530 nm.

2.13. Effect of photoilluminated riboflavin on respiratory chain lactate dehydrogenase activity

The LDH activity was measured by evaluating the reduction of NAD⁺ to NADH and H⁺ as lactate is oxidized to pyruvate [25]. The treated bacterial cell suspension was centrifuged at 4 °C for 30 min at 300 g and pellet was collected in a microplate. To the collected pellet LDH reaction solution was added and the sample was incubated for 30 min at 25 °C. The OD was read at 490 nm.

2.14. Growth inhibition assay by CFU

Survival of bacteria in all the bacterial suspension groups was determined by counting the numbers of colony forming units (CFU) [26].

2.15. Live and dead evaluation by light microscopy

The treated bacterial samples were collected after centrifugation and suspended in PBS. Methylene blue stock was prepared by dissolving 0.1 mg/ml in sodium citrate solution. Sample and dye were mixed in a ratio of 1:1 and incubated for 5 min at room temperature, followed by visualization on an optical microscope (Olympus).
2.16. Morphological evaluation by scanning electron microscopy

The effect of photoilluminated riboflavin on E. coli’s cellular morphology was determined by scanning electron microscope (SEM) [27]. The treated bacterial cells were centrifuged at 4000 rpm. The collected pellet was fixed with 2.5% glutaraldehyde for 20 min. The samples were then centrifuged and resuspended in PBS and about 20 μl of sample was placed on a glass slide. The samples on the slide were dehydrated using a graded series of alcohol (30%, 50%, 70%, 90%, and 100%). After the final step of drying, slides were observed under the scanning electron microscope.

2.17. Statistical analysis

Experimental values were expressed as mean ± S.E.M. of three independent experiments. All experiments were statistically analyzed by one-way analysis of variance. P < 0.05 were considered statistically significant.

3. Results

3.1. Riboflavin gets excited under photoillumination and generates superoxide radical

Upon photoillumination, riboflavin gets excited and degrades as evident from loss of its absorption maxima after 2h. of incubation under white light (Fig. 1a) whereas there is no change in absorption maxima without photoolillumination (Fig. 1b). NBT assay of riboflavin also depicted significant superoxide radical production under photoolillumination (Fig. 2a) whereas no considerable radical generation in absence of light (Fig. 2b). These results clearly show significant photosensitive nature of riboflavin.

3.2. Photoilluminated riboflavin generate significant intracellular ROS and induces oxidative stress

As seen in Fig. 3a E. coli cells when incubated in presence in riboflavin alone, showed no significant ROS generation, however when cells were incubated with riboflavin and irradiated with white light for 2h. significant ROS generation was observed. Also, no considerable ROS generation was observed when cells were exposed to white light alone (Fig. 3a). Assessment of lipid peroxidation is one of the prominent biomarkers of oxidative stress. As seen from Fig. 3b the level of MDA in E. coli cells treated with riboflavin in presence of light is significantly higher as compared to MDA levels present in E. coli cells treated with riboflavin and light separately. This indicates that riboflavin, when illuminated with light, induces oxidative stress in bacterial cells.

3.3. Photoilluminated riboflavin alters redox status of E. coli

To determine the physiological impact of ROS generated as a result of irradiation of riboflavin on bacterial cells, we measured the activity of ROS related enzymic and non-enzymic parameters namely SOD, catalase, GST, and GSH. As evident from our data, the activity of SOD (Fig. 4a) and catalase (Fig. 4b), significantly reduced in samples which are exposed to photoilluminated riboflavin as compared to samples which are exposed to riboflavin without light and exposed to light alone. The decrease in the level of cellular antioxidant metabolite GSH was significantly reduced in samples exposed to photoilluminated riboflavin (Fig. 4d). To further validate these observations, we measured the activity of GST. GST is a GSH utilizing enzyme primarily involved in detoxification process. As evident from Fig. 4c, the specific activity of GST enzyme was increased significantly in cells exposed to photoilluminated riboflavin.

3.4. Oxidative stress induced by photoilluminated riboflavin burst respiratory chain and causes bacterial death

LDH is a mitochondrial enzyme which readily gets denatured under oxidative stress conditions and is a reliable protein to evaluate the ROS generated damaging effect on the respiratory system of the bacterial cell [28]. As shown in Fig. 5a the LDH activity in cells treated with photoilluminated riboflavin is considerably reduced as compared to riboflavin and light treated cells separately. As shown in Fig. 5b the colony number in the bacterial group exposed to photoilluminated riboflavin is significantly low as compared to the number of colonies present in the bacterial group exposed to riboflavin or light separately. This shows that photoilluminated riboflavin induces oxidative stress which burst respiratory chain leading to the denaturation of LDH enzyme as well as ultimately succumbing bacterial cells to death.

3.5. Analysis of live and dead bacteria using optical and scanning electron microscopy

Methylene blue staining is a conventional and reliable method to analyze bacterial cell death. Its fundamental lies on the fact that live bacteria appears unstained due to the enzymatic reduction of the dye into a colorless product, on the other hand, dead cells appear blue due to the staining [29]. As shown in Fig. 6a, the bacterial sample which was treated with riboflavin and exposed to light exhibited blue stain (panel D) under optical microscope, whereas, samples exposed to riboflavin (panel C) or light (panel B) separately were similar to control live bacterial sample (panel A) not exhibiting blue stain. As evident from the obtained scanning electron microscopic images (Fig. 6b), clear disrupted membrane integrity and altered cellular morphology were

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**Fig. 1.** Absorption spectra of riboflavin (50 μM) (a) Rf illuminated for 2 h in white light (b) Rf without the illumination of light.
observed in the cells treated with photoilluminated riboflavin (panel D). The control cells (panel A) and the cells treated with light (panel B) and riboflavin (panel C) alone showed no significant change in cellular morphology. Thus, it was confirmed that riboflavin under photoillumination induces bacterial cell death.

4. Discussion

Nosocomial infections pose a serious threat to the patient’s health and recovery and involve a lot of expenditure in their control by the hospital. Among these infections, the hospital-acquired infections caused by gram-negative bacteria draws major concern because these microbes are highly efficient in acquiring antibiotic resistance with a plethora of different mechanisms targeting existing antibiotics [5]. Development of safe antibiotics is the only remaining option to combat this hospital-acquired health hazard and therefore insights into this area have been a concern for a considerable time [30].

Antimicrobial photodynamic therapy represents an excellent therapeutic modality against drug-resistant microbes [31]. An optimal photosensitizer is pre-requisite for efficient photodynamic activity. Riboflavin on account of having different meso-atoms possess excellent photo-physical and photochemical properties [32]. This study adds another dimension to the use of photoactivated riboflavin, demonstrating that clinically important multi-drug resistant bacteria are susceptible to ROS generated by photoactivated riboflavin. Significant NBT reduction by riboflavin in presence of light (Fig. 2) confirmed photosensitivity of this vitamin whereas increased DCF level in the bacterial sample treated with photoilluminated riboflavin (Fig. 3a) showed significant intracellular ROS generation. Our results are supported by studies from our lab [33–35] and those of others [36,37] that photoactivated riboflavin does produce ROS. Riboflavin absorbs photons from the illuminated light and undergoes intersystem conversion i.e. from singlet state to triplet state. This triplet state reacts with molecular oxygen, thereby producing reactive oxygen species. Thus, we can say that this generated ROS attacks cellular macromolecules and disintegrates membrane integrity, thereby, killing the organism (Fig. 7).

The altered level of cellular antioxidants (Fig. 4), decreased LDH activity (Fig. 5a) and lipid peroxidation (Fig. 3b) confirmed significant

![Fig. 2. NBT Assay: Time-dependent in vitro superoxide radical production by (a) Rf (50 μM) under photoillumination (b) without photoillumination. Sodium phosphate buffer (50 mM, pH 8) was used as a control. Values reported are mean ± S.E.M. of the three independent experiments.](image1)

![Fig. 3. (a). DCFH- DA assay to determine ROs generation by photoilluminated riboflavin inside E. coli cell. (b). Lipid peroxidation: as measured by assaying TBA adducts formed. Values reported are mean ± S.E.M. of the three independent experiments. *p < 0.5 as compared to control E. coli cells.](image2)
macromolecular damage, which is in accordance with previous reports of macromolecular damage due to extensive ROS production in bacterial cells [38,39]. Thus, it can be suggested that due to extensive macromolecular damage the \textit{E. coli} cells are drifted towards cellular death as evident from colony forming data (Fig. 5b). The obtained optical microscopic (Fig. 6a) and the electron microscopic images (Fig. 6b) further validated the toxic effect of photoilluminated riboflavin. Our results can be correlated with previous findings of antibiotic lethality due to altered redox status [40]. The effect of ROS scavengers on oxidative damage induced by photoilluminated riboflavin generated oxygen radicals has already been explored previously in our lab [34,41] and it has been clearly observed that if any protein/compound/enzyme is added that can quench generated ROS, then the detrimental effect of ROS is inhibited. Therefore, if any ROS quencher will be present in solution the bacterial inactivation will be hindered. This provides a clear evidence that the exhibited antibacterial effect is irrefutably ROS mediated.

It has not escaped our notice that the mechanism used by photoactivated riboflavin does not target any protein(s) and/or a sequence but uses a fundamental chemical property of riboflavin to generate ROS. This is particularly important since most mechanisms of drug resistance [42] are mediated by specific genes and gene products and therefore chances of the bacterial cell evolving ahead of antibiotic development may possibly be ruled out in our case as the target of ROS is randomly selected. Therefore, we can say that photoilluminated riboflavin is an efficacious in vitro approach to target nosocomial infection-causing microbes.

4.1. Conclusion

We propose a resistance proof photodynamic mechanism using riboflavin as a photosensitizer to target bacterial nosocomial infections and conclude our work in anticipation of developing photodynamic therapy as a first line therapy for combatting nosocomial infectious...
health hazard.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Fig. 6. (a) Methylene blue-stained treated E. coli samples (panel A) E. coli; (panel B) E. coli + light; (panel C) E. coli + riboflavin and (panel D) E. coli + light + riboflavin. All samples were incubated for two hrs. before white light. (b) SEM images of treated E. coli samples (panel A) E. coli; (panel B) E. coli + light; (panel C) E. coli + riboflavin; (panel D) E. coli + light + riboflavin. All samples were incubated for two hrs. before white light and were fixed in 2.5% glutaraldehyde.

Fig. 7. Riboflavin upon light exposure undergoes intersystem conversion from singlet state to the triplet state. The triplet state reacts with available O2 atom and generates oxygen radicals. These generated radicals cause membrane damage leading to bacterial death.

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