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Silibinin protects *Staphylococcus aureus* from UVC-induced bactericide *via* enhanced generation of reactive oxygen species

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Silibinin is a major bioactive component of silymarin extracted from the milk thistle *Silybum marianum*. Silibinin has therapeutic potential for a wide variety of applications including anticancer, hepatoprotective and antiinflammatory medicines. There are studies reporting that silibinin has shown anti-bacterial effects, but its underlying mechanism has not yet been elucidated. In the present study, UVC inhibited growth of *S. aurues* in a dose-dependent manner and up-regulated production of reactive oxygen species (ROS). Silibinin treatment improved the survival of *S. aureus* in the presence of UVC. Interestingly, silibinin further enhanced the generation of ROS and activities of antioxidant enzymes (catalase (CAT) and glutathione peroxidase (GSH-PX)). To determine the role of ROS induced by silibinin, the scavengers (*N*-acetylcysteine (NAC), glutathione (GSH) and superoxide dismutase (SOD)) or donors (*t*BHP and H_2O_2) of ROS were used to treat the bacterial cells. The results showed that ROS scavengers down-regulated the protective effect of silibinin, while ROS donors up-regulated it. Therefore, ROS produced by silibinin protects *S. aureus* cells from UVC-induced cell death. Our findings revealed novel insights into the relationship between silibinin, bacteria and ROS. Elucidation of the relationship will contribute to the development of important applications for further use of natural products, particularly for therapeutic strategies for *S. aureus*-associated diseases.

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Introduction

Silibinin is a popular dietary supplement and has a long history of hepatoprotective use by humans, exhibiting extremely low toxicity.[1,2] In previous studies, silibinin was shown to have antibacterial effects against *Bacillus subtilis* and *Staphylococcus epidermidis* through inhibiting synthesis of macromolecules such as RNA and proteins.[3] Silibinin and antibiotics (ampicillin and oxacillin) had a synergistic bactericidal effect on methicillin-resistant *Staphylococcus aureus* isolated from clinical specimens.[4]

UV irradiation induces DNA damage, such as formation of cyclobutane pyrimidine dimers (CPD).[5,6] UV suppresses the transcription of target genes and induces apoptosis if unrepaired in hamster cells.[7] UV is divided into three regions according to spectrum wavelength: UVA (320–400 nm), UVB (280–320 nm) and UVC (200–280 nm). Among them, UVC has the strongest bactericidal effect.

ROS are toxic byproducts of aerobic metabolism. Other stresses such as nutrient limitation and ultraviolet radiation also elicit oxidant responses.[8,9] Depending on molecular sensing and efficient signaling to enhance antioxidant defense mechanisms such as eliminating/decomposing ROS or repairing the ROS-caused DNA damage, cell survival and resistance to oxidative stress are enhanced.[10–12] ROS can interact with RNA, DNA, proteins and lipids.[13] The cell's repairing machinery such as superoxide dismutases and catalases can regulate endogenously generated ROS and maintain them in normal level.[12] On other hand, at higher concentrations, ROS could induce oxidative stress that changes the pattern of gene expression, generating more protein indirectly or directly involved in ROS scavenging or in repairing ROS-caused DNA damage.[12,14]

In recent years, it has been proposed that ROS are highly correlated with the cause of bacterial death.[13,15] Antibiotics (norfloxacin, ampicillin and kanamycin) induce ROS by activating the electron transport chain and kill *Escherichia coli* (*E. coli*) by causing destabilization of cell structures.[16] Conversely, recent report declared that ROS enhanced cephalosporin

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resistance in *Enterococcus faecalis*.[17] Briefly, ROS have ambivalent effects on bacteria, leading to death or survival, implying that we need further clarification of the mechanisms that ROS affect cell life.

Many natural products and chemicals with a modulating effect on ultraviolet (UV)-induced ROS generation have been well reviewed.[18] However, there are few reports on the relationship among silibinin, bacteria and ROS. Thus, in this study we investigated bioactive effects of silibinin on UVC-treated *S. aureus*.

In contrast to the effect of silibinin on antibiotics-induced cell death, here we found that silibinin at an optimal concentration showed a cytoprotective effect from UVC-induced cell death. ROS played an important role in this protective effect of silibinin.

Materials and methods

Bacterial strains and culture conditions

Staphylococcus aureus JCM2413^T were cultured overnight on a rotary shaker (220 × *g*) in LB (Luria–Bertani)-rich medium (1% tryptone, 0.5% yeast extract and 1% NaCl) at 37 °C. The cells were stored at 4 °C in LB agar.

Reagents

Silibinin (99% purity) was obtained from Jurong Best Medicine Material (Zhenjiang, Jiangsu, China). It was dissolved in dimethylsulfoxide (DMSO) to make a stock solution. *N*-acetylcysteine (NAC), glutathione (GSH), superoxide dismutase (SOD), hydrogen peroxide (H_2O_2), *tert*-butylhydroperoxide (*t*BHP) and 2′,7′-dichlorofluorescein diacetate (DCFDA) were purchased from Sigma Aldrich (St Louis, MO, USA). The kits of CAT and GSH-PX were from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

UVC exposure and silibinin treatment

The bacteria grown to the stationary phase (24 h) were harvested by centrifugation at 14 000 × *g* (10 min), followed by resuspension in fresh LB medium. Cell suspensions 0.5 mL volume were placed in sterile glass Petri dish 45 mm diameter (ensuring a bacterial monolayer) and exposed to different doses of UVC (135–1080 J m^{-2}) with gentle shacking at room temperature. Then, the cells were cultured at 37 °C for 12 h. The radiation dose was adjusted by a UVC spectra radiometer (Lin technology Company, Shenzhen, China), with emission of UVC radiation ranging from 230 to 280 nm with a peak at 254 nm. The cells were irradiated with UVC in the presence or absence of silibinin.

Colony forming unit (CFU) assay

The survival ratios of *S. aureus* were determined by bacterial colony forming units (CFU). Cells subjected to the indicated treatments were collected, diluted in LB to a density of 10^9, and 200 μL aliquots were spread-plated (at least in triplicates) in LB medium solidified with 1.5% agar. Colonies were counted after incubation at 37 °C overnight.

Flow cytometry of DCF-DA positive cells

Intracellular production of ROS was detected using the probe 2′,7′-dichlorofluorescein diacetate (DCFDA).[19] Cells subjected to the indicated treatments were collected, washed with 10 mM potassium phosphate buffer (pH 7.0), adjusted with the probe (final concentration 10 μM), and incubated at 37 °C for 60 min in dark. DCFDA-stained cells were analyzed by using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Enzyme activity assay

Catalase (CAT) and glutathione peroxidase (GSH-PX) activities were determined with commercial kits according to the manufacturer's instructions. CAT activity was measured spectrophotometrically by monitoring the ratio of decomposition of H_2O_2.[20] One unit of CAT was defined as the amount required to decompose 1 μmol of H_2O_2 per minute under the assay conditions. The GSH-PX activity was based on the principle that oxidation of glutathione (GSH) was catalyzed by GSH-PX to produce oxidized glutathione (GSSG). Decrease in the concentration of GSH that reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) yielding stable yellow substances with absorbance at 412 nm is indicative of GSH-PX activity in cells.[21]

Statistical analysis

All the experiments were conducted at least in triplicates ($n \geq 3$). Data were expressed as the mean ± standard deviation (SD). One-way repeated analysis of variance (ANOVA) and Tukey–Kramer multiple-comparison test were used to the determine a statistical significance. GraphPad 7.0 was used for all statistical calculations. A p value of <0.05 was considered statistically significant.

Results and discussion

UVC irradiation reduced cell viability of *S. aureus* dose-dependently

After exposure to UVC irradiation, the numbers of *S. aureus* colonies were substantially reduced in a dose-dependent manner (Fig. 1A). CFU analysis also demonstrated statistically significant decreases in the bacterial loads of *S. aureus* after the irradiation (Fig. 1B). These results showed the bactericidal effects of UVC exposure on *S. aureus*. The biological effects of UV are usually attributed to enhanced production of ROS, which results in oxidative damage to lipids,[22] proteins[23] and DNA.[24] Therefore, we moved to determine the production of ROS by monitoring the fluorescence intensity after DCFH-DA staining. Results showed that UVC increased ROS production to the maximum at 270 J m^{-2} (Fig. 1C).

Silibinin protected *S. aureus* cells from UVC-induced cell death

Silymarin flavonolignan is a complex mixture of polyphenolic molecules in which silibinin is the major bioactive component.[25,26] Silibinin and silymarin both showed antibacterial activities.[27] Silymarin demonstrated antimicrobial activity

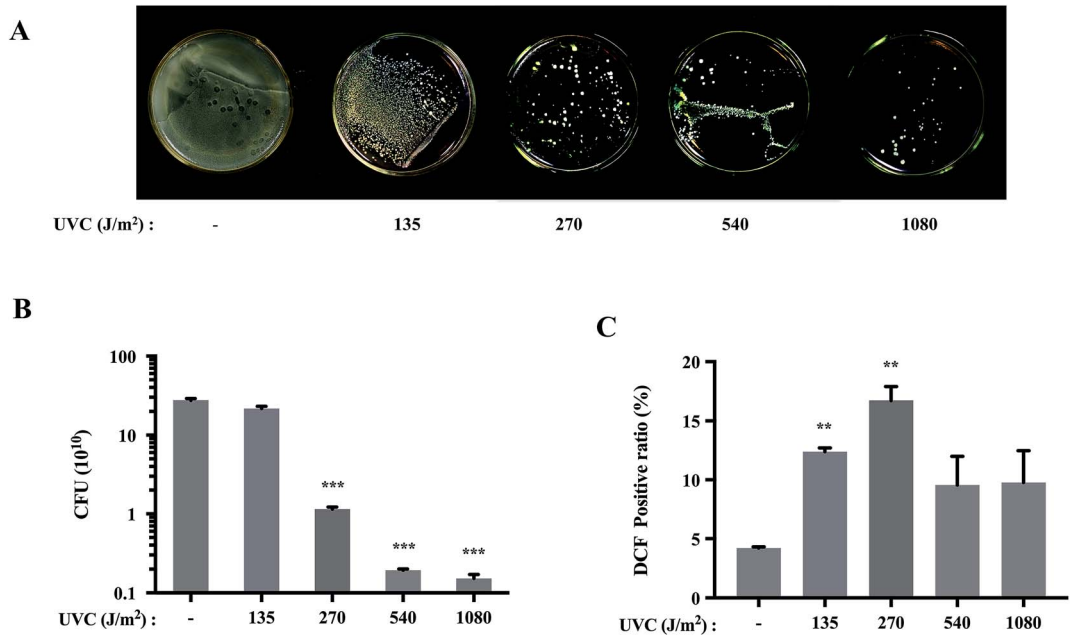

Fig. 1 UVC induced the dose-dependent reduction in the viability of *S. aureus* cells. (A) Representative images of *S. aureus* following treatment with different doses of UVC. (B) CFU of *S. aureus* treated with different doses of UVC irradiation. (C) *S. aureus* cells were irradiated with different doses of UVC, re-incubated and analyzed for ROS production after 12 h. (**$p < 0.01$ *vs.* control group). Date are means value ± SD from three independent experiments. (***$p < 0.001$ *vs.* control group).

against *Candida albicans* with a minimum inhibitory concentration (MIC) of 512 μg mL^{-1}. Meanwhile, silibinin showed potent bactericidal activity against *E. coli* with a MIC of 133 μM.[27] Silibinin inhibited RNA and protein synthesis in *Bacillus subtilis* and *Staphylococcus epidermidis* at the concentration of 417 μM.[3]

Interestingly, the present investigation provides a new role of silibinin: silibinin blocks UVC-induced *S. aureus* cell death. Silibinin treatment alone on *S. aureus* did not affect cell viability (Fig. 2A and B). However, UVC-induced *S. aureus* cell death was attenuated to varying degrees by silibinin administration. Silibinin exhibited the maximal effect on cell survival at 25 μM. At higher concentration (200 μM), silibinin did not reverse UVC-induced bactericidal effect (Fig. 2D and E). Silibinin plays a protective role at 25–50 μM, while it exerts bactericidal effect at concentrations higher than 100 μM.[3,27] Silibinin possibly possesses bi-directional impacts on both eukaryotic and prokaryotic cells, depending on the concentrations. In human hepatocellular carcinoma cell line HepG2 cells, at a concentration of 200 μM, silibinin induces DNA lesions, generates oxidized DNA bases and reduces cell viability. However, between 10 and 100 μM, silibinin was able to reduce the genotoxic effect induced by bleomycin, benzopyrene or aflatoxinB_1.[28]

It has been well documented that many polyphenols including silibinin are known to have antioxidant activity, but they can also be turned into oxidants under certain conditions.[29] For example, silibinin enhances ionizing radiation-induced ROS production under prolonged oxidative stress in

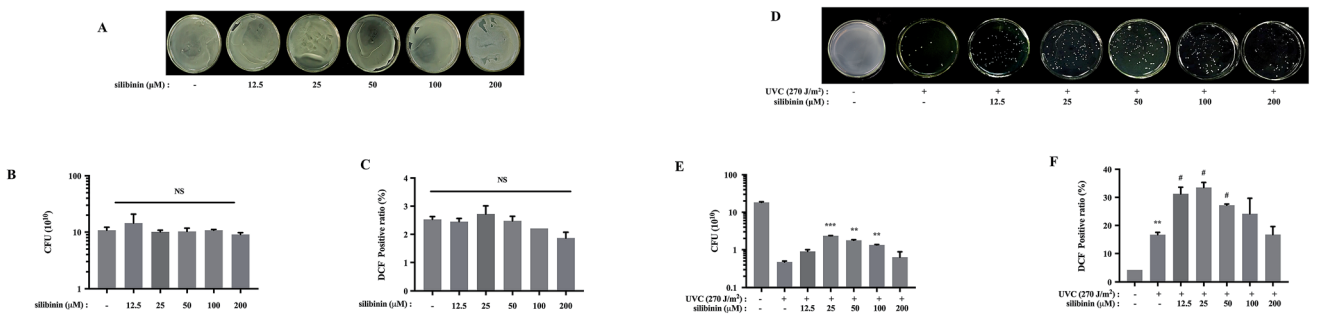

Fig. 2 Silibinin was effective in protecting *S. aureus* cells forming UVC-induced cell death. (A) Images of *S. aureus* cultured following silibinin treatment at the indicated concentrations for 12 h. (B) Results of CFU in silibinin-treated *S. aureus*. (C) Effects of silibinin on ROS generation. (NS: nonsignificant). (D) Representative images of silibinin-treated *S. aureus* cultured following UVC irradiation. (E) CFU were measured after UVC irradiation in the absence or presence of silibinin. Date are means value ± SD from three independent experiments. (**$p < 0.01$, ***$p < 0.001$ *vs.* UVC-irradiated group). (F) UVC-irradiated *S. aureus* cells were post-treated with silibinin, harvested, and analyzed for ROS production. (**$p < 0.01$ *vs.* control group; #$p < 0.05$ *vs.* UVC-irradiated group).

human prostate cancer cells.[30–32] We examined the effect of silibinin on the ROS production of *S. aureus*, in the absence or presence of UVC. Silibinin did not increase ROS levels in *S. aureus* cells by itself (Fig. 2C), but it significantly enhanced ROS generation in the presence of UVC (Fig. 2F). The ROS production was dependent on silibinin dosage, increasing initially and decreasing later with a maximal enhancement with silibinin at a concentration of 25 μM. This trend was consistent with the protective efficacy of silibinin on UVC-treated *S. aureus* cells, suggesting a possible relationship between ROS production and cell protection with silibinin treatment.

Scavenging of ROS by NAC, GSH or SOD reduced the protective effect of silibinin on UVC-irradiated *S. aureus*

Programmed cell death is well established in eukaryotes. It is suggested to exist in bacteria.[33–35] UVC irradiation, a kind of non-ionizing one, can induce oxidative stress, causing eukaryotic apoptosis.[36] Some results suggested that UVC-induced bacterial cell death was primarily due to ROS-mediated DNA damage.[37–39] Classically ROS were considered as deleterious agents, contributing to a vast range of damage in eukaryotes or prokaryotes. Consistently, in this study, high concentrations of ROS were deleterious to living cells, leading to cell death. Our results are consistent with the previous research that *E. coli* growth inhibition was caused by a production of ROS by silver nanoparticles.[40] However, many studies implicate that ROS could function in the process of signal-transduction pathways to regulate transformation and uncontrolled growth potential of tumor cells.[41,42] According to the work by Lubart and co-workers, low amounts of ROS generation could promote bacterial proliferation.[43]

In order to test whether ROS production was involved in the protective effect of silibinin on UVC-induced bacterial cell death, the effects of ROS scavengers were examined. It is known that NAC and GSH reduce endogenous ROS and counteract oxidative stress.[44,45] GSH contains a thiol group (–SH) which is highly reactive and is the only one substrate of selenium-dependent GSH peroxidase (GSH-PX),[46] and NAC is a precursor of GSH.[47] SOD, an antioxidant enzyme, scavenges the excessively generated ROS, superoxide anions in particular, protecting *Beauveria bassiana* from damage.[48] Viability of *S. aureus* cells was not affected by the treatment with NAC, GSH or SOD alone, as shown by the CFU assay. However, NAC, GSH and SOD down-regulated the protective effect of silibinin in UVC-irradiated *S. aureus* (Fig. 3A, B, E, F, I and J), suggesting a protective role of the ROS

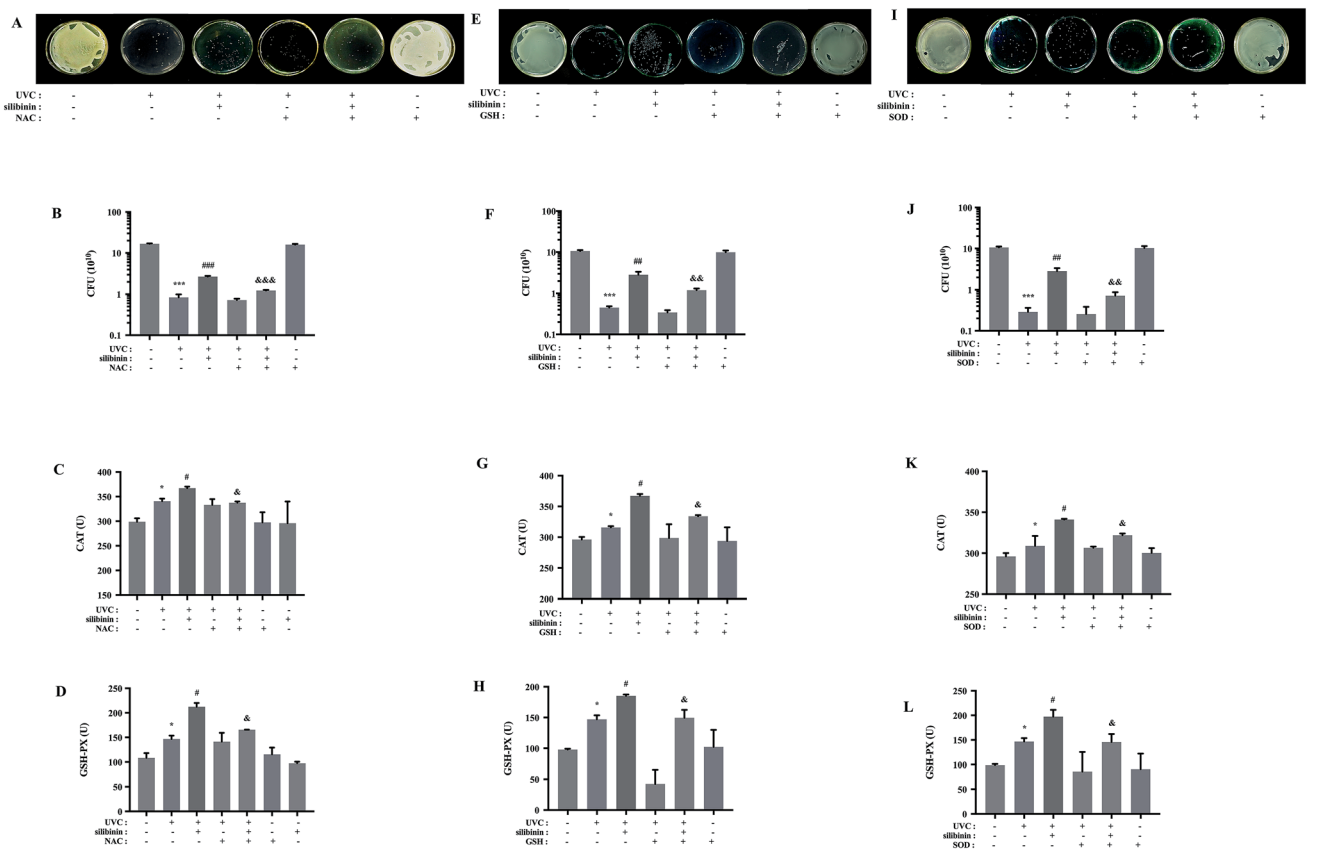

Fig. 3 Scavenging of ROS by NAC, GSH or SOD reduced the protective effect of silibinin on UVC-irradiated *S. aureus*. In the absence or presence of NAC (8 mM), GSH (10 mM) and SOD (75 U), UVC-irradiated *S. aureus* were treated with silibinin. Images of representative cultures of *S. aureus* treated with NAC, GSH or SOD. CFU were determined (A and B, E and F, I and J). (***$p < 0.001$ *vs.* control group; ##$p < 0.01$, ###$p < 0.001$ *vs.* UVC-irradiated group; &$p < 0.05$, &&$p < 0.01$, &&&$p < 0.001$ *vs.* silibinin plus UVC-irradiated group). Changes of the activity of CAT (C, G and K) and GSH-PX (D, H and L) in *S. aureus*. (*$p < 0.05$ *vs.* control group; #$p < 0.05$ *vs.* UVC-irradiated group; &$p < 0.05$ *vs.* silibinin plus UVC-irradiated group). Date are means ± SD from three independent experiments.

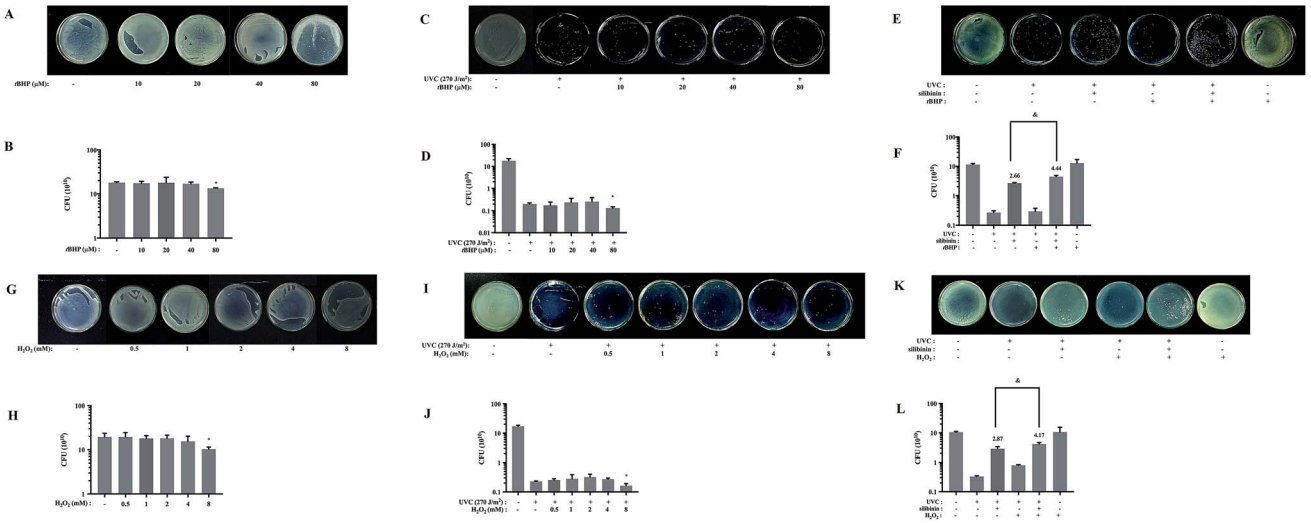

Fig. 4 Silibinin might enhance ROS level, protecting *S. aureus* from UVC irradiation. Images of cultures as follow *S. aureus* were treated with *t*BHP from 10 to 80 μM or H_2O_2 from 0.5 to 8 mM were shown (A and G) and CFU were determined (B and H). (*$p < 0.05$ *vs.* control group). (C and I) Representative images of *t*BHP- or H_2O_2-treated *S. aureus* cultured, following UVC irradiation. (D and J) CFU were measured after UVC irradiation with or without of *t*BHP or H_2O_2. (*$p < 0.05$ *vs.* UVC-irradiated group). In the absence or presence of H_2O_2 or *t*BHP, UVC-irradiated *S. aureus* were treated with silibinin. Representative images of cultured *S. aureus* with different treatments. CFU assays were conducted (E, F, K and L). (&$p < 0.05$ *vs.* silibinin plus UVC-irradiated group). Date are means ± SD from three independent experiments.

induced by silibinin treatment. NAC, GSH and SOD repressed the activities of catalase (CAT) and GSH-PX elevated by silibinin treatment, indicating a decreased oxidative stress (Fig. 3C, D, G, H, K and L). Therefore, we speculated that some kinds of ROS at a localized region in cells or for a time exerted the protective roles in UVC-treated *S. aureus* cell death.

Silibinin might enhance ROS level, protecting *S. aureus* from UVC irradiation

*t*BHP is an organic hydroperoxide which has been usually employed to induce oxidative stress in various biological systems.[49] H_2O_2 is one type of ROS. High concentrations of ROS can cause the death of microorganisms.[50] As Fig. 4A–D and G–J shows that in the absence or presence of UVC irradiation, both 80 μM *t*BHP and 8 mM H_2O_2 significantly reduced *S. aureus* growth ratio. But when the UVC-irradiated bacteria were treated with lower concentrations of *t*BHP (10 and 40 μM) or H_2O_2 (ranged from 0.5 to 4 mM), the growth ratio did not significantly change (Fig. 4D and J). Moreover, the growth ratio in 20 μM *t*BHP-treated group (4.4 ± 0.4060) was significantly higher than that in silibinin alone group (2.66 ± 0.1155) in UVC-treated *S. aureus* (Fig. 4E and F). Treatment with 1 mM H_2O_2 also increased the growth ratio to about 1.5 folds (4.17 ± 0.5657) of that treated with only silibinin (2.87 ± 0.5033) in UVC-irradiated *S. aureus* (Fig. 4K and L). These results suggested that silibinin and *t*BHP/H_2O_2 have a synergistic protective effect. High levels of ROS induced oxidative stress, leading to bacterial cell death. However, the certain concentration of ROS may play a potentially protective role in *S. aureus*.

UVC irradiation induces the formation of various reactive oxygen species.[8,51] Low concentration of H_2O_2 could play a crucial role in regulation of cell metabolism and cellular signaling in response to environmental stresses such as low- or medium-pressure UV irradiation.[52] Our results demonstrated that H_2O_2 enhanced the protective effect of silibinin on UVC-induced *S. aureus* cell death. We speculated that silibinin protects UVC-irradiated bacteria from cell death *via* enhancing production of some kinds of ROS. But the precise identification of these molecules and their mechanisms requires further investigation.

Mitochondria are evolved from endosymbiotic α-proteobacteria belonging to *Rickettsia* gender.[53,54] They still present many similarities to prokaryotic cells such as a double membrane, and the ability to produce ATP through the generation of a proton gradient generated across the inner membrane.[53,54] As toxic byproducts of aerobic metabolism, ROS are primarily formed in mitochondria and peroxisomes, but also at any other cellular compartments. They are then removed or detoxified by an array of antioxidative enzymes such as CAT and SOD, and antioxidants. Many antioxidative systems of the cells, therefore, keep ROS at a basal non-toxic level.[55] In eukaryotes, mitochondrial oxidative stress leads to cell death, as 5-aminolevulinic acid enhances ionizing irradiation-induced mitochondrial oxidative stress and leads to increased glioma cell death accompanying changes of mitochondrial morphology.[56] However, mitochondrial oxidative stress also activates cell survival signaling.[57] Silibinin induces excess mitochondrial ROS production, resulting in increased survival of human melanoma A375-S2 cells.[58] The effect of ROS in bacteria and mitochondria might potentially share some similar regulation mechanisms through ROS.

Conclusions

Oxidative stress has been implicated as one of the mechanisms whereby UVC kills bacteria. The formation of ROS may play

distinct roles in bacteria killing and survival. The present study provides new facts that silibinin-induced ROS are beneficial to the survival of *S. aureus* under UVC-irradiated conditions. The study uncovers novel insights into the relationships between silibinin and bacteria, which has important implications for the usage of natural products in development of therapeutic strategies for *S. aureus*.

Acknowledgements

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