Accepted: 27 November 2017

DOI: 10.1111/jocd.12488

ORIGINAL CONTRIBUTION

WILEY JCD Journal of Cosmetic Dermatology

Antioxidant action and protective and reparative effects of lentinan on oxidative damage in HaCaT cells

Yusha Zi MS | Bo Zhang BS | Biao Jiang MS | Xingyao Yang BS | Zilu Liang BS | Weiyi Liu BS | Congfen He PhD | Lei Liu PhD

Beijing Key Laboratory of Plant Resources Research and Development, School of Science, Beijing Technology and Business University, Beijing, China

Correspondence
Lei Liu, Beijing Key Laboratory of Plant Resources Research and Development, School of Science, Beijing Technology and Business University, Beijing, China.
Email: liulei@btbu.edu.cn

Funding information
Science and Technology Planning Project of Beijing, Grant/Award Number: Z171100001517006; the Research Foundation of Beijing Technology and Business University, Grant/Award Number: LKJJ2016-11

Summary

Background: *Lentinus edodes* is one of the largest edible fungi. Lentinan, extracted from its fruiting body has clinically significant anticancer, antibacterial, antiviral, and anticoagulant effects; however, its preventive effects on skin oxidative damage are unclear.

Aims: We aimed to evaluate the in vitro antioxidation capability of lentinan and its protective and reparative effects on a model of cell oxidative damage.

Methods: We evaluated the in vitro antioxidant potential of lentinan by assessing its free-radical quenching ability using DPPH and ABTS and superoxide anions. Using the HaCaT cell line as the experimental system, we tested the protective and reparative effects of lentinan on a model of H_2O_2-induced cellular oxidative damage through assessment of cell survival rate, malondialdehyde (MDA) content, and superoxide dismutase (SOD) activity.

Results: Lentinan displayed high antioxidant potential: DDPH and ABTS quenching rates were above 60%; superoxide anions, approximately 18%. Furthermore, lentinan could dose-dependently prevent the reduction of activity in HaCaT cells by H_2O_2, reduce MDA formation, and increase SOD activity. Moreover, lentinan showed not only a protective effect against oxidative damage but also reparative effects to a certain extent, in HaCaT cells.

Conclusions: Our findings demonstrated the ability of lentinan to enhance cellular tolerance to oxidative damage, stress resistance, and to have protective and reparative effects on damaged cells. Therefore, with *L. edodes* as a source for antiaging substances, cosmetics with homology to foods have great potential clinical applications.

KEYWORDS
HaCaT cells, hydrogen peroxide, lentinan, oxidative damage

1 | INTRODUCTION

The skin is the largest organ, being the outermost integument in humans. In the course of skin metabolism, oxidative stress gradually damages the skin and external environmental contaminants and ultraviolet rays cause oxidative damage.[1] Reactive oxygen species (ROS), primarily superoxide anions, hydrogen peroxide, free radicals such as hydroxyl radicals, and nonfree radicals are significant mediators during oxidative damage processes.[2] In particular, H_2O_2, a prominent ROS, inhibits cell proliferation and causes oxidative damage to important cellular macromolecules such as lipids and DNA,[3,4] thereby resulting in severe biological consequences such as cell aging

and death, mutation, and even cancer.[5] H_2O_2 has long been used widely to establish cellular models of acute oxidative stress to investigate the mechanisms underlying free-radical-mediated cellular damage; such an approach is important to understand reparative and protective mechanisms for oxidative damage.

Lentinus edodes (phylum Basidiomycota, family Pleurotaceae) is one of the largest edible fungi and the most popular and widely cultivated edible medicinal fungus[6] referred to as "Mountain Delicacy" in the folk society. To date, *L. edodes* plantations have been industrialized in more than 120 countries.[7] The primary ingredients of dry *L. edodes* are proteins, fats, carbohydrates, minerals, and vitamins, and among them, polyoses play a deterministic role in the biological activity of *L. edodes*.[8,9] Lentinan is extracted from high-quality *L. edodes* fruiting body, and has been reported to have antitumor, anticancer, antiviral, anticoagulant, and immunological effects.[10] Chen et al[11] reported that lentinan can quench free hydroxyl radicals and superoxide anions and can chelate Fe^{2+}. Recently, lentinan has been globally accepted as an alternative medicinal or food supplement.[12] However, few studies have investigated the role of lentinan in preventing oxidative damage to the skin.

This study aimed to investigate the in vitro antioxidant potential of crude hot water lentinan extracts by measuring the quenching rates of DPPH and ABTs free radicals and superoxide anions. Based on the free-radical quenching rates thus obtained, we measured cellular survival rate, malondialdehyde (MDA) content, and superoxide dismutase (SOD) activity via a protection route (ie, adding the sample first and then damaging the sample) and via a repair route (ie, damaging the sample first and then repairing the sample) in the human immortalized keratinocyte cell line, HaCaT. The findings of this study may aid the development of protective and reparative cosmetics with lentinan as an antiaging substance.

2 | MATERIALS AND METHODS

2.1 | Materials

Lentinan 808 (Oriental Nutri-woods, China); HaCaT cells (National Infrastructure of Cell line Resource, China); DMEM culture medium (HyClone, SH30243.01); pancreatin (Gibco,25200-056); fetal bovine serum or FBS (HyClone,SV30087.02); 3% H_2O_2 (I009, Nanjing Jiancheng Biotechnology Ltd., China); cck-8 kit (C0038, Beyotime Biotechnology, China); MDA kit (A003-1, Nanjing Jiancheng Bioengineering Institute, China); total SOD activity kit (S0101, Nanjing Jiancheng Biotechnology Ltd., China); DPPH (*Pinellia ternata*, B2689-1); pyrogallol (*Pinellia ternata*, b2030); ABTS (*Pinellia ternata*, b2422-1).

2.2 | Determination of antioxidant potential in vitro

2.2.1 | Assessment of DPPH free-radical quenching

DPPH free-radical quenching was assessed on the basis of the method used by Li et al,[13] with minor modifications, as elaborated below. One milliliter of lentinan solution (0.01% VC was used as positive control) and 1 mL of an ethanolic solution of 2×10^{-4} mol/L of DPPH were mixed homogeneously (tube A1). One milliliter of absolute ethanol and 1 mL of an ethanolic solution of 2×10^{-4} mol/L DPPH were mixed homogeneously (tube A2). One milliliter of distilled water and 1 mL of sample solution (VC) were mixed homogeneously (tube A3). After allowing the reaction to proceed in the dark for 30 minutes, the absorbance of mixtures in tubes A1, A2, and A3 was measured at 517 nm, with the quenching rate (%) defined as $([A2 + A3] - A1)/A2$.

2.2.2 | Assessment of superoxide anion quenching

One milliliter of Tris-HCl buffer solution was incubated in a 25°C water bath for 20 minutes, followed by addition of a 0.5 mL lentinan sample solution and a 0.4 mL 3 mmol/L pyrogallol solution to form a mixture under rapid agitation to ensure a complete reaction (pyrogallol was replaced by 0.4 mL 3 mmol/L HCl in group B). After allowing the reaction to proceed for 4 minutes, a 0.1 mL 3 mmol/L HCl solution was used to terminate the reaction. The absorbance was measured at 325 nm, with the quenching rate calculated as $(A0 - A1 + A2)/A0 \times 100\%$. Here, A0 was the absorbance of the aforementioned reaction-quenched mixture with pure water replacing the sample solution, A1 was the absorbance of sample solution, and A2 was the absorbance of the aforementioned reaction-quenched mixture with 10 mmol/L HCL replacing pyrogallol.

2.2.3 | Assessment of ABTS free-radical quenching

ABTS free-radical quenching was assessed on the basis of the method used by Li et al[14] with minor modifications. An ABTS working solution was prepared by mixing equal volumes of a 7.4 mmol/L ABTS solution and a 2.6 mmol/L potassium persulfate solution. After allowing the reaction to proceed in the dark for 12 hours to ensure that ABTS was completely quenched, the solution was diluted continually with absolute ethanol. Absorbance was measured at 734 nm and maintained at 0.7 ± 0.02 after the subtraction of blank absorbance. Thereafter, 0.8 mL of ABTS working solution was fully mixed with 0.2 mL of lentinan sample solution or 0.01% VC and allowed to settle still for 6 minutes, and then the absorbance of the resulting mixture was measured at 734 nm. The quenching rate was calculated as $(A0\text{-}A1)/A0 \times 100\%$. Here, A0 was the absorbance of the aforementioned mixture with pure water replacing the sample solution, and A1 was the absorbance of the sample solution.

2.3 | In vitro cellular analysis of lentinan

2.3.1 | Culturing of HaCaT cells

HaCaT cells have an unlimited proliferative capacity, are easy to obtain and culture, and are well capable of epidermal differentiation.[15] HaCaT cells were cultured in DMEM with 10% FBS at 37°C and 5% CO_2. HaCaT cells in the logarithmic growth stage were

harvested by aspirating the culture media from the culture flasks and rinsing them 1-2 times with PBS, followed by addition of 1 mL of trypsin to disrupt cellular adhesion at 37°C under 5% CO_2 for 8 minutes. When 80%-90% detachment, trypsinization was terminated with a 1:2 trypsin:culture media. After centrifugation at 200 *g* at 25°C for 4 minutes, the supernatant was discarded, and the cells were resuspended in a certain amount of culture medium for subculturing at a later stage or for cell count plating.

2.3.2 | Experimental grouping

Blank control group: culturing for 28 hours in culture medium with 10% PBS; H_2O_2 model group: After culturing for 12 hours, 3 mg/mL H_2O_2 was added to act on the cells for 4 hours, followed by culturing for another 12 hours; protection group: After culturing for 12 hours, lentinan solutions of different concentrations were added to the culture medium for another 12-hour culturing period, followed by addition of H_2O_2 to stimulate the cells for 4 hours; repair group: After culturing for 12 hours, H_2O_2 was added to the medium and allowed to act on the cells for 4 hours, and then lentinan solutions of different concentrations were added for another 12-hour culturing period.

2.3.3 | Cell survival rate analysis

Cell survival rate was determined using the cck-8 kit. HaCaT cells were inoculated at a density of 1×10^5 cells/mL in 96-well plates, followed by addition of 10 μL of cck-8 in each well and allowing the reaction to proceed for 4 hours, after which the optical density (OD) was measured at 450 nm to determine cell survival rates.

2.3.4 | Determination of MDA content and SOD activity

After the aforementioned grouping, 3 mg/mL H_2O_2 diluted with the culture medium was added to damage the cells for 4 hours, followed by repeated freezing and thawing until cell membranes were ruptured; thereafter, the test solutions were collected. Malondialdehyde (MDA) content and SOD activity were measured in accordance with the manufacturer's instructions.

2.4 | Statistical analysis

All the data were processed using Graphad Prism 6.0 software, with the experimental results expressed as mean ± standard deviation (SD). An intergroup *t* test was performed, and $P < .05$ was considered significant; $P < .01$, extremely significant.

3 | RESULTS

3.1 | In vitro antioxidation of lentinan

Lentinan displayed high antioxidant potential and quenched DPPH (quenching rate, 0.094-1.5 mg/mL) and ABTS (quenching rate, 23.44-375 μg/mL) free radicals and superoxide anions (quenching rate, 23.44-375 μg/mL) at different concentrations, in a concentration-dependent manner (Figure 1).

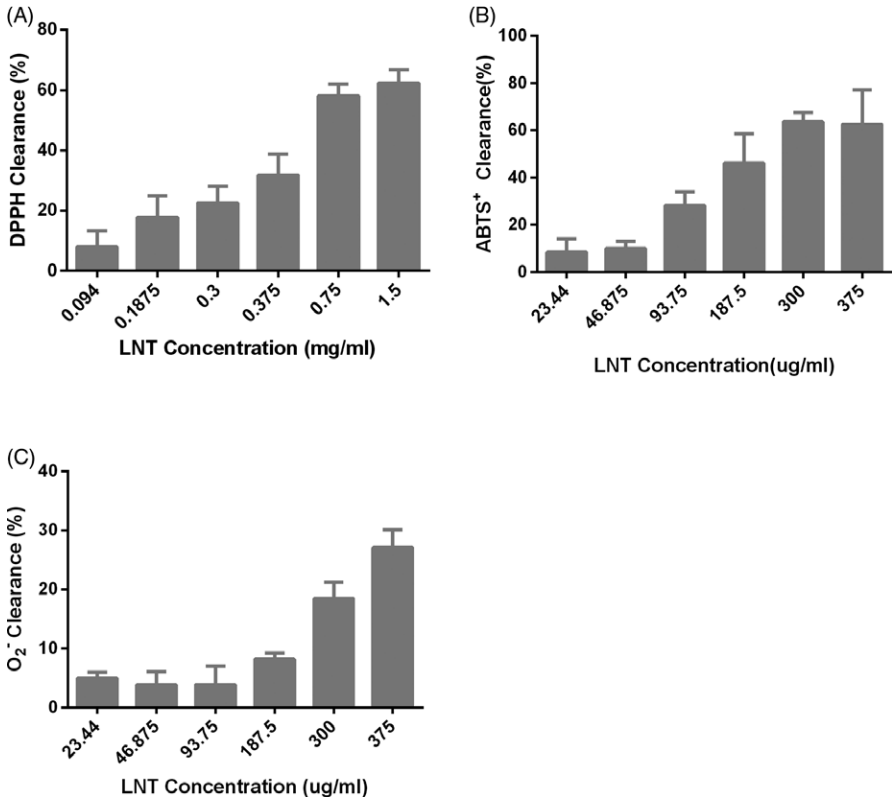

FIGURE 1 The antioxidant potential of lentinan at different concentrations. Scavenging effects of lentinan on DPPH free radicals (A), ABTS free radicals (B), and superoxide anion free radicals (C) with 0.1% ascorbic acid as a positive control. Results are expressed as the mean ± SD (n = 3)

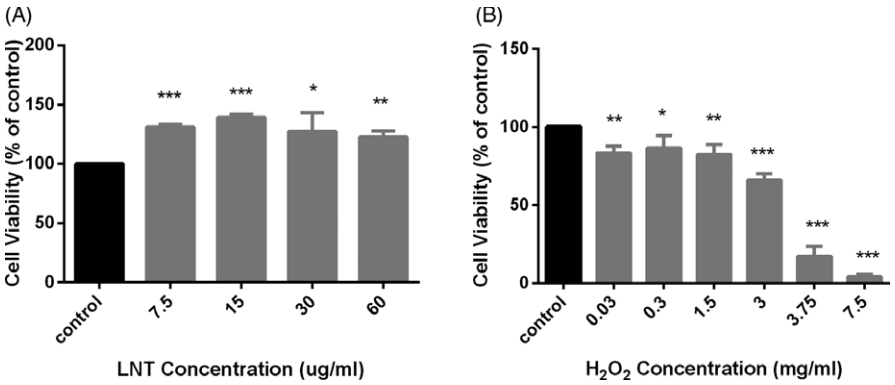

FIGURE 2 Cell viability determined using the cck-8 assay. A, Effects of lentinan on the viability of HaCaT cells. B, Cells were incubated with of 0.03-7.5 mg/mL H_2O_2 for 28 h. Results are expressed as the mean ± SD (n = 3). The Student's *t* test was performed to determine statistical significance (*$P < .05$, **$P < .01$, ***$P < .001$)

3.2 | Effects of lentinan on the activity of H_2O_2-damaged HaCaT cells

First, the toxicity of lentinan to HaCaT cells was measured with the cck-8 kit, as illustrated in Figure 2A. After 7.5, 15, 30, and 60 μg/mL lentinan was added in the medium of HaCaT cells for 12 hours, the survival rate varied in a concentration gradient-dependent manner ($P < .05$). After oxidative damage in HaCaT cells induced by 0.03-7.5 mg/mL H_2O_2, the survival rate showed a significant concentration gradient-dependence in comparison with the blank control group ($P < .05$, $P < .01$; Figure 2B). The 3 mg/mL H_2O_2 was selected for further use.

3.3 | Protection of H_2O_2-damaged HaCaT cells by lentinan

3.3.1 | Effects of protection group lentinan on the activity of H_2O_2-damaged HaCaT cells

After 3 mg/mL H_2O_2 was added in the media of cells for 4 hours, the survival rate of HaCaT cells decreased significantly($P < .01$), whereas the preadded lentinan at different concentrations rescued cells from H_2O_2-induced cellular damage in a concentration-dependent manner (Figure 3A). Moreover, there was a significant difference between the protection group with 6 μg/mL lentinan and the H_2O_2 model group ($P < .01$). When the concentration of protection group decreased to less than 0.3 μg/mL, the cell activity was similar to that in the normal control group ($P > .05$).

3.3.2 | Effects of lentinan on MDA content and SOD activity in H_2O_2-damaged HaCaT cells in the protection group

Compared with the blank control group, the MDA content in the H_2O_2 model group was significantly elevated ($P < .01$) and the SOD activity decreased significantly ($P < .01$). After lentinan was added at different concentrations for 24 hours and then 3 mg/mL H_2O_2 was added in the culture media of the cells for 4 hours, the 1.5, 3, and 6 μg/mL lentinan solutions significantly decreased MDA content ($P < .01$; Figure 3B) and a significant increase in SOD activity in the protection group ($P < .01$; Figure 3C).

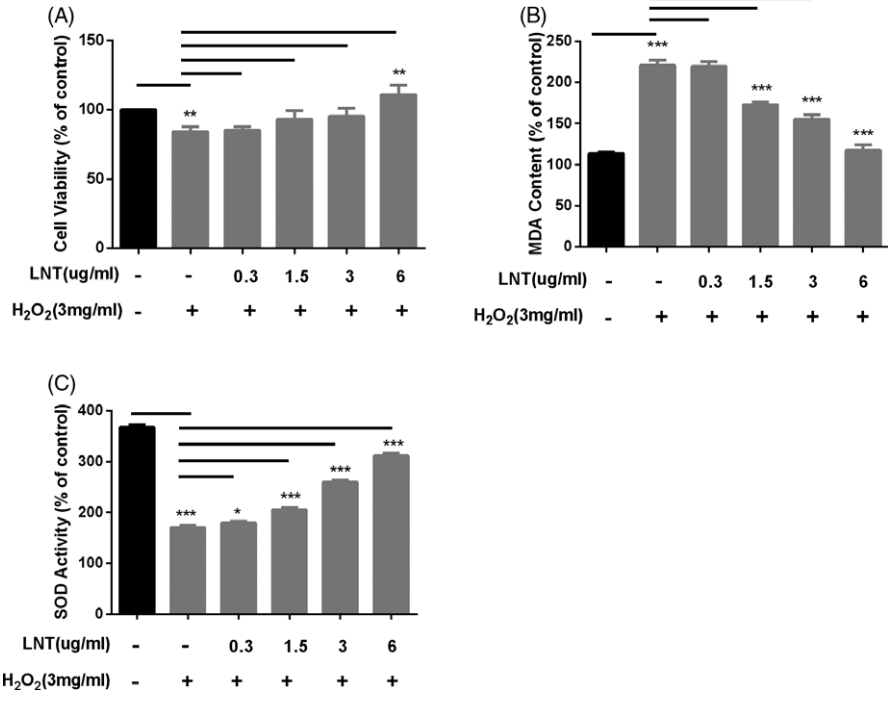

FIGURE 3 T1he protective effect of lentinan pretreatment on H_2O_2-induced HaCaT cells damage. A, Cells were treated with 3 mg/mL H_2O_2 only or pretreated with 0.3, 1.5, 3 and 6 μg/mL lentinan solutions followed by 3 mg/mL H_2O_2 for 4 h. B, Effects of lentinan on malondialdehyde (MDA) content of H_2O_2-damaged HaCaT cells. C, Effects of lentinan on superoxide dismutase (SOD) activity of H_2O_2-damaged HaCaT cells. Cells were pretreated 0.3, 1.5, 3 and 6 μg/mL lentinan solutions 12 h prior to incubation with 3 mg/mL H_2O_2. Results are expressed as the mean ± SD (n = 3). The Student's *t* test was performed to determine statistical significance (*$P < .05$, **$P < .01$, ***$P < .001$)

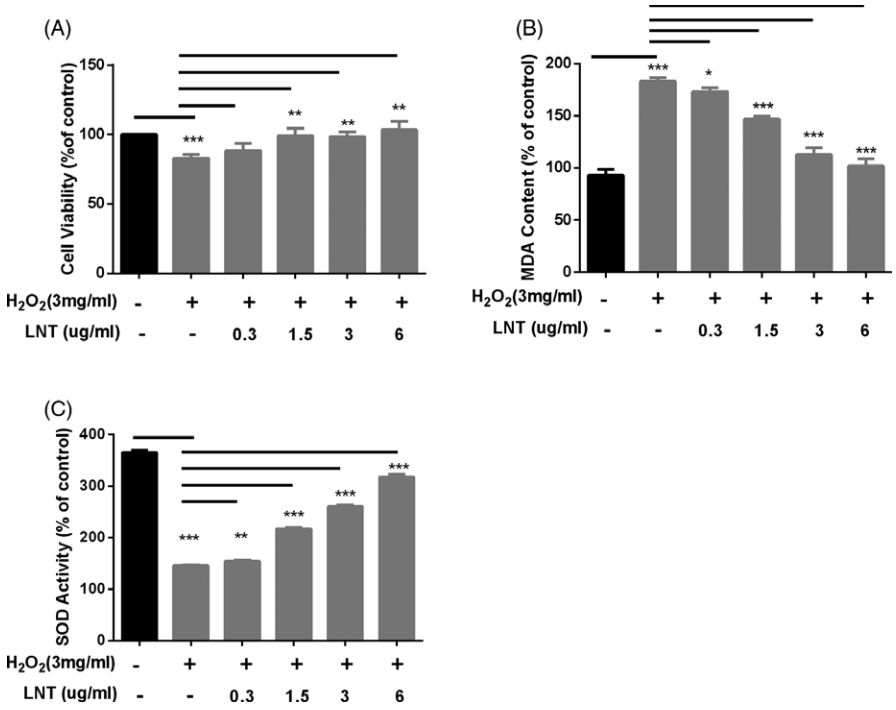

FIGURE 4 The reparative effect of lentinan pretreatment on H_2O_2-induced HaCaT cellular damage. A, Cells were treated with 3 mg/mL H_2O_2 only or pretreated with 3 mg/mL H_2O_2 for 4 h prior to 0.3, 1.5, 3 and 6 μg/mL lentinan solutions. Effects of lentinan on the (B) malondialdehyde (MDA) content and (C) superoxide dismutase (SOD) activity of H_2O_2-damaged HaCaT cells. Results are expressed as the mean ± SD (n = 3). The Student's *t* test was performed to determine the statistical significance (*$P < .05$, **$P < .01$, ***$P < .001$)

3.4 | Reparative effect of lentinan on H_2O_2-damaged HaCaT cells

3.4.1 | Effect of lentinan on the activity of H_2O_2-damaged HaCaT cells in the repair group

Lentinan, at different concentrations, yielded good cellular repair (Figure 4A), with the repair group containing 1.5, 3, and 6 μg/mL lentinan and significantly differing from the H_2O_2 model group ($P < .01$); however, there was no significant difference in cell activity between the repair group containing 0.3 μg/mL lentinan and the control group ($P > .05$), in the H_2O_2-damaged HaCaT cells.

3.4.2 | Effect of lentinan on MDA content and SOD activity in H_2O_2-damaged HaCaT cells in the repair group

In the H_2O_2 model group, MDA content was significantly elevated ($P < .01$), while the SOD activity decreased significantly ($P < .01$). After the stimulation to HaCaT cells with 3 mg/mL H_2O_2 for 4 hours, lentinan addition at concentrations of 0.3, 1.5, 3 and 6 μg/mL for 12 hours significantly decreased MDA content ($P < .01$; Figure 4B) and elevated the SOD activity ($P < .01$; Figure 4C).

4 | DISCUSSION

With advancements in the modern sciences, cosmetics have advanced from basic skin care products focusing on the cleaning and moisturizing of the skin to functional cosmetics focusing on delaying of skin aging and improving the skin appearance. Recently, more studies have focused on plant-derived natural biological macromolecules such as proteins, polypeptides, and polyoses with lentinan being one of the latter.[16] Lentinan is reported to have good skin care activity such as beautifying, whitening, and moisturizing of the skins as well as showing antisensitive and anti-microbial effects;[17,18] however, few studies have investigated the antiaging effects of lentinan on the skin.[19]

The free radical theory proposed by Denham in 1956 stated that free radicals are strongly capable of oxidizing unsaturated lipids in biological membranes to form lipid peroxides (with MDA as the final product), which constitute a type of strong cross-linking agent reacting with proteins and nucleic acids to form insoluble substances that harden biological membranes, thereby decreasing their permeability and affecting intracellular transport of materials; hence, these cells are functionally inactive and undergo apoptosis.[20] Metabolism of many substances generates free-radical peroxides to maintain free radicals in nonequilibrium states. An excess of free radicals is likely to cause damage to the body. When damage due to radicals accumulates beyond the body's repair capacity, they lead to a change or even loss of cell differentiation states, thereby resulting in skin aging.[21] Reactive oxygen species (ROS) are reported as one of the causes inducing and promoting human aging.[22] Therefore, the elimination of oxygen free radicals, that is, antioxidation, is an effective antiaging method.

In this study, to investigate the effect of crude hot water extracts of lentinan as a functional raw material for antiaging cosmetics, we conducted biochemical investigations in a cellular model, wherein quenching of DPPH (quenching rate, 0.094-1.5 mg/mL) and ABTS (quenching rate, 23.44-375 μg/mL) free radicals and superoxide anion free radicals (quenching rate, 23.44-375 μg/mL) by lentinan was found to increase in a concentration-dependent manner, thereby indicating the high free-radical quenching capacity of

lentinan. The mechanism underlying lentinan antioxidation may involve a hydrogen-migration scheme in which hydrogen atoms are transferred to other molecules to terminate the chain reactions of free radicals while converting them to harmless substances.[23] In particular, superoxide anions play a very important role, as they may induce the formation of other free radicals, thereby resulting in damage to cell membranes and oxidative degradation of nucleic acids, in turn leading to chronic damage and, moreover, the occurrence of many other closely associated diseases. Therefore, elimination of superoxide anions can prevent ROS-induced tissue damage and delay aging. Moreover, superoxides are also considered a consequence of indirectly induced lipid peroxidation.[24]

To better reflect the actual metabolism of the body, we further employed a cellular model of oxidative damage to determine the antioxidant potential of lentinan. H_2O_2 has long been used widely to establish cellular models of acute oxidative stress, for investigating free-radical-induced damage to cells.[25,26] The experiments were conducted with the HaCaT cell line, derived from the outermost layers of human epidermis, and established an H_2O_2-induced cellular damage model.[27] The 3-mg/mL concentration of H_2O_2 decreased the survival rate of cultured HaCaT cells and their SOD activity decreased significantly, while their MDA content in the culture medium was elevated significantly.

We categorized the action of lentinan on H_2O_2-damaged HaCaT cells into protection vs repair, as they correspond to two types of potential applications. For instance, functional raw materials with a protective effect can be used to prevent and delay skin aging, and functional raw materials with a reparative effect can be used for skin recovery after oxidative damage. Our experiments showed that in both groups, lentinan could significantly enhance the activity of H_2O_2-damaged HaCaT cells. Some animal experiments have shown that subcutaneous injection of lentinan can significantly promote lymphocyte proliferation.[28] Moreover, in both groups, lentinan significantly increased SOD activity and decreased MDA content. SOD is the primary enzymatic defense system against free radicals in the body, and increased SOD activity indicates that the body is undergoing antioxidation to prevent free-radical-induced damage and decrease cell damages.[29] Lentinan has been recently reported to significantly increase SOD activity in the blood in animals, so as to inhibit ROS and free radical synthesis, thereby making lentinan antioxidative.[30] However, MDA is the product of lipid peroxidation reactions between free radicals and polyunsaturated fatty acids in biological membranes, and its content reflects the level of oxygen free radicals and the degree of lipid peroxidation,[31] thereby indirectly representing the degree of cell damage. Some studies have reported that lentinan can significantly decrease MDA content in neurons and prevent them from undergoing lipid peroxidation.[32] As evident from the present findings, it was possible to use lentinan to decrease the degree of lipid peroxidation in the body and MDA content in H_2O_2-damaged HaCaT cells also decreased. However, owing to numerous bioactive groups in the ingredients of lentinan, further studies are required to elucidate what ingredients and mechanisms play a role in antioxidation.

5 | CONCLUSION

This study investigated the antioxidative activity of lentinan, and revealed the string free-radical-quenching capacity of lentinan and their quenching rates, in a concentration-dependent manner. Lentinan showed a dual effect—protection and repair—on H_2O_2-damaged HaCaT cells. The cellular experiments showed that lentinan could significantly increase the activity of H_2O_2-damaged HaCaT cells and SOD activity, decrease MDA content, inhibit lipid peroxidation, and effectively protect HaCaT cells from oxidative damage as well as repair damaged HaCaT cells, thereby supporting further studies on lentinan antioxidation and showing revealing implications for antioxidation-based delaying of skin aging.

ACKNOWLEDGMENTS

This study was supported by Science and Technology Planning Project of Beijing (Z171100001517006) and the Research Foundation of Beijing Technology and Business University (LKJJ2016-11).

ORCID

Congfen He http://orcid.org/0000-0001-5944-4472
Lei Liu http://orcid.org/0000-0003-3943-6381

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How to cite this article: Zi Y, Zhang B, Jiang B, et al. Antioxidant action and protective and reparative effects of lentinan on oxidative damage in HaCaT cells. *J Cosmet Dermatol*. 2018;17:1108–1114. https://doi.org/10.1111/jocd.12488