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Microwave-assisted synthesis of chitosan biguanidine hydrochloride and its antioxidant activity *in vitro*

Qianwen Liu,[1,2,3] Yuan Li,[1,2,3] Xin Jin,[1,2,3] Qin Peng,[1,2,3] Xiaofei Liu,[1,2,3] Yuntang Wu[4]

[1]Department of Polymer Materials Science and Engineering, College of Materials Science and Engineering, Tianjin University, Tianjin, 300072, People's Republic of China

[2]Research Institute of Advanced Polymer, Tianjin University, Tianjin, 300072, People's Republic of China

[3]Tianjin Key Laboratory of Composite and Functional Materials, Tianjin University, Tianjin, 300072, People's Republic of China

[4]Department of Health Statistics, College of Public Health, Tianjin Medical University, Tianjin, 300070, People's Republic of China

Correspondence to: X. F. Liu (E-mail: liuxf315@aliyun.com)

ABSTRACT: Chitosan biguanidine hydrochloride (CSGH) was synthesized by guanidinylation of chitosan hydrochloride (CSH) under microwave irradiation and characterized by Fourier transform infrared spectroscopy, gel permeation chromatography, and element analysis. The antioxidant activity of CSGH was evaluated by both chemical and cellular methods. The results showed that CSGH was better than CSH but slightly inferior to ascorbic acid (Vc) for scavenging 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radicals, and the DPPH• scavenging ability of CSGH was dose-dependent. However, cell experiments indicated CSGH had a superior repairing effect on oxidatively damaged cells. The intervention of CSGH could excellently recover the cell morphology, decrease the level of malondialdehyde, and enhance the activity of superoxide dismutase and glutathione peroxidase. CSGH could be a potential antioxidant reagent, and its antioxidant activity was reflected not only in scavenging the free radicals but also in regulating the oxidation/antioxidation balance of damaged cells. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43570.

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INTRODUCTION

In the physiological activities of the human body, oxygen, functioning as an important nutritional factor, may produce highly reactive oxygen free radicals, more generally known as "reactive oxygen species" (ROS) and "reactive nitrogen species" (RNS), which are closely related to a series of pathological and physiological phenomena such as proliferation, differentiation, and apoptosis.[1,2] Excess ROS and RNS play critical roles in inducing undesirable consequences, involving problems of aging,[3] tumors,[4] cerebral injury,[5] and diabetes[6] and its complications.[7]

Chitosan, formed primarily of repeating units of β-(1→4)-2-amino-2-deoxy-D-glucose (D-glucosamine), was proved to possess good biocompatibility and broad-spectrum antibacterial function and has been shown to be involved in plenty of beneficial health effects.[8–10] Our previous report also demonstrated that chitosan and its derivatives with proper molecular weights and doses could significantly increase the activity of superoxide dismutase (SOD) and decrease the level of malondialdehyde (MDA) in the serum, liver, and kidney of obese rats.[11]

On the other hand, a guanidine compound, which contains the $NH_2C(=NH)NH-$ group, has recently drawn great attention because of its wide variety of activities, including antitumor effects,[12] hypoglycemic activity,[13] and enhancement of cellular uptake ability.[14]

In recent years, some studies have reported the synthesis of chitosan and guanidine compounds, focusing on their antibacterial capacity,[15–18] but the antioxidant activity of such synthetic products has not been mentioned. Hu *et al.*[19] proved guanidinylated chitosan derivatives to show much better antibacterial activity than chitosan, but the substitution degrees were not high because of the decrease of the pH value during the reaction process.

Compared to traditional bath heating, microwave irradiation, a new method for supplying heat to chemical reactions, has become more and more popular because of its advantages of controllability, faster heating, higher thermal energy utilization and sensitivity, and more uniform heating.[20–22] Zhao *et al.*[23] prepared chitosan biguanidine hydrochloride under microwave irradiation. Because the reaction solvent was a solution of hydrochloric acid, the molecular weight of the guanidinylated

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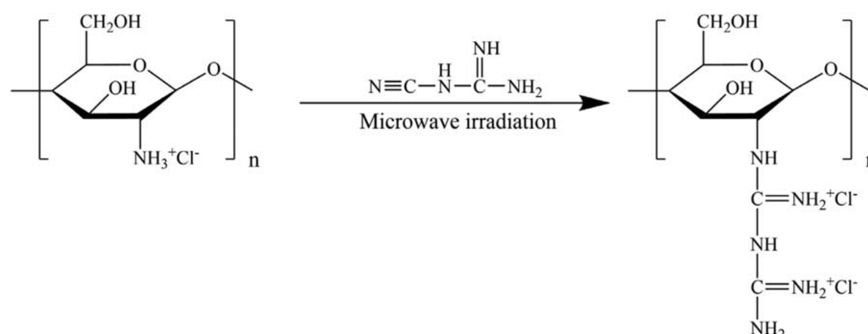

Figure 1. Synthesis of CSGH.

chitosans decreased with increasing microwave irradiation time because the polymer backbone degraded under strong acid conditions.

In this paper, different from the previous reports, we prepared chitosan biguanidine hydrochloride by first preparing chitosan hydrochloride and then reacting with dicyandiamide in a water solution under microwave irradiation. The antioxidant ability was evaluated by both chemical and cellular methods in an attempt to explore the potential antioxidant value of chitosan biguanidine hydrochloride.

EXPERIMENTAL

Materials

Chitosan [weight-average molecular weight (M_w) of 5.0×10^4 with a degree of deacetylation (DD) of 93.2%] was provided by Qingdao Medicine Institute (Shandong, China). Dicyandiamide, ascorbic acid (Vc), and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO). The human hepatocellular carcinoma cell line HepG2 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS), and trypsin were provided by Gibco-BRL (Grand Island, NY). All the kits used were provided by Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Other chemicals were of analytical grade and were provided by Jiangtian Chemical Technology (Tianjin, China), and ultrapure water was used throughout the experiments.

Preparation of CSGH

Raw chitosan was thoroughly dissolved in 0.2 mol/L HCl solution and precipitated with ethanol. The precipitated solid was washed with anhydrous ethanol, followed by filtration, vacuum drying, and grinding to give the chitosan hydrochloride (CSH) powder.

A 2.0 g portion of CSH powder dissolved in 100 mL distilled water was added into a four-neck flask. Dicyandiamide was introduced into the CSH solution (at 2:1 ratio of dicyandiamide to CSH) followed by stirring until thorough dissolution. The flask was placed in a microwave reactor (Biaohe Instrument, Shanghai, China), and the reaction was run with stirring for 10 min at 90 °C (see reaction equation in Figure 1). The microwave power was set to 400 W while working. The reaction temperature was traceable by a temperature detector and controllable by automatic intermittent work of the microwave reactor. After it was cooled to room temperature, the mixture was concentrated by reduced-pressure distillation. The product chitosan biguanidine hydrochloride (CSGH) was obtained by dialysis for three days in a dialysis bag with molecular weight cutoff of 5000 followed by lyophilization. For comparison purposes, the dicyandiamide solution was microwaved without CSH using the same microwave setting and postprocessing.

Characterization of CSGH

Fourier Transform Infrared Analysis. The Fourier transform infrared (FTIR) spectrum of CS and CSGH was scanned at room temperature over the wavenumber range 4000–400 cm^{-1} using a Spectrum 100 FTIR spectrometer (Perkin-Elmer, Fremont, California, USA) via the KBr pressed-disc method.

Potentiometric Titration. The degree of substitution (DS) of CSGH was determined by potentiometric titration,[24,25] which was based on NaOH reacting with the $=NH_2^+\ Cl^-$ group in CSGH. An abrupt change of pH at the isoelectric point was captured, and the DS was calculated by using the following formulas [eq. (1)–(3)], where Δv represents the titrant volume, C_{NaOH} represents the concentration of NaOH, n_1 and n_2 represent the mole number of substituted and unsubstituted constitutional units, respectively, and G represents the sample mass:

$$n_1 = \Delta v \times C_{NaOH} \times 10^{-3}/2 \quad (1)$$

$$n_2 = (G-318 \times n_1)/161 \quad (2)$$

$$DS\ (\%) = [n_2/(n_1+n_2)] \times 100 \quad (3)$$

Elemental Analysis. Elemental analysis was performed with a Vario Micro cube element analyzer (Elementar, Germany). The DS of CSGH was calculated again by the following formula [eq. (4)]:

$$C(\%)/N(\%) = [6 \times 12 + 2 \times 12 \times (1-DD) + 2 \times 12 \times DS]/(14 + 4 \times 14 \times DS) \quad (4)$$

Gel Permeation Chromatography Analysis. The molecular weights (M_w) of CSGH and CSH were determined with a Waters (Milford, Massachusetts, USA) 600E gel permeation chromatograph (GPC), in which 0.25 M $NaNO_3$ was used as the eluent with a flow rate of 0.5 mL/min at 45 °C.

DPPH• Radical Scavenging Ability

CSH, CSGH, and Vc were dissolved in water at various concentrations (0, 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 4.8, 6.4, and 8.0 mg/mL) as the sample solutions.

A 500 µL portion of the sample solution was thoroughly mixed with 500 µL of a 0.20 mM DPPH-ethanol solution. After reaction for 30 min in the dark, the absorbance of the mixture (A_{sample}) at 515 nm was measured with an RG-3000 microplate reader (Bio-Tek, Winooski, Vermont, USA). Meanwhile, the absorbance of a mixture of ethanol (500 µL) and DPPH-ethanol solution (500 µL) was measured as A_{DPPH}, and ethanol (500 µL) with the sample solution (500 µL) as A_{blank}. The calculation formula is as follows [eq. (5)]:

$$\text{DPPH} \cdot \text{Scavenging Activity}(\%) = [1-(A_{sample}-A_{blank})/A_{DPPH}] \times 100 \tag{5}$$

The half maximal effective concentrations (EC50) of Vc, CSH, and CSGH were estimated according to the line chart.

Cell Experiments

Cell Culture and Treatments. HepG2 cells were cultured in DMEM supplemented with 15% FBS in a cell culture flask at 37 °C in a humidified atmosphere of 5% CO_2. The culture media were refreshed every other day.

To establish the model of oxidative damage, cells at the logarithmic growth phase were treated with H_2O_2 at a final concentration of 50 µM for 2 h.

The HepG2 cells re-treated with H_2O_2 were further supplemented with 100 µL of the intervening solution, according to which three groups were set up: Group CSGH-L, CSGH-M, CSGH-H (representing CSGH at 60, 300, 600 µg/mL in DMEM solution, respectively); Group CSH-L, CSH-M, CSH-H (representing CSH at 60, 300, 600 µg/mL in DMEM solution, respectively); and Group Vc-L, Vc-M, Vc-H (representing Vc at 60, 300, 600 µg/mL in DMEM solution, respectively). Instead of the intervening solution, group N (normal HepG2 cells untreated with H_2O_2) and group D (damaged HepG2 cells treated with H_2O_2) were treated with 100 µL of DMEM solution as control. The interference was conducted at 37 °C for 24 h in a humidified atmosphere of 5% CO_2. For each group at least eight wells were used.

Cell Viability Assay. A 15 µL portion of 5mg/mL methylthiazoletetrazolium (MTT) was added into each well and incubated for 4 h. Then the nutrient solution was removed and 200 µL of DMSO was added to each well. After vibration for 30 min on an orbital shaker to dissolve the crystals completely, the absorbance was measured at 490 nm with a SUNRISE microplate reader (Tecan, Männedorf, Switzerland).

Cell Morphology. The normal HepG2 cells and damaged ones treated with H_2O_2, as well as those further interfered with CSGH, CSH, or Vc at 300 µg/mL in DMEM solution, were dyed and observed under an SZ61 phase-contrast microscope (Olympus, Tokyo, Japan).

Measurement of Antioxidant Indexes. The protein concentration was assayed by the method of Coomassie brilliant blue.[26] The level of MDA was measured by thiobarbituric acid assay,[27] which is based on the reaction of MDA with thiobarbituric acid (TBA). The absorbance of the colored reaction product was measured at 532 nm. The resulting MDA level was expressed as nmol per mg protein.

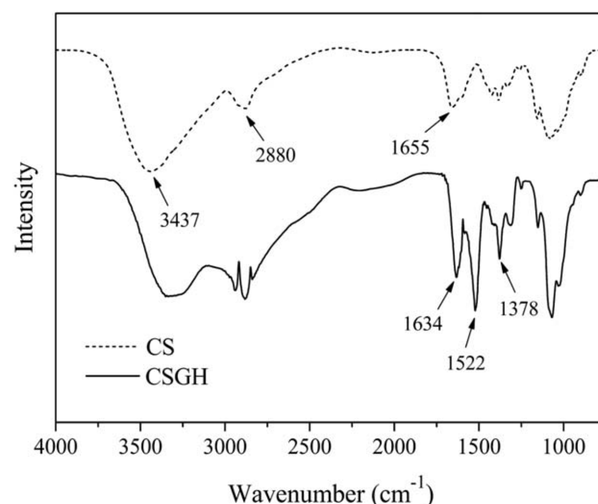

Figure 2. FTIR spectra of CS and CSGH.

SOD and GSH-Px activities were assessed using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The method of SOD analysis was based on the superoxide radicals generated by the reaction of xanthine and xanthine oxidase, which was inhibited by SOD. One unit (U) of SOD was generally defined as the amount of enzyme that inhibits superoxide radicals by 50%. After addition of the chromogenic agent, the absorbance was measured at 550 nm, and the activities of SOD were expressed as U per mg protein. The method of GSH-Px analysis was based on the colored product of the GSH-Px reaction with 5,5′-dithio-bis-(2-nitrobenzoic acid). Its absorbance was detected at 412 nm, and the activities of GSH-Px were expressed as U per mg protein.

Statistical Analysis

The results are shown as mean ± standard deviations (SDs). Means were compared by one-way analysis of variance, with the different groups compared by Fisher's protected least-significant difference (LSD) test, and a value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

FTIR

Microwaved dicyandiamide was dialyzed in a dialysis bag with molecular weight cutoff 5000 and gave no product after lyophilization; that is, the reaction conditions in the present experiment did not generate any product from dicyandiamide only.

The structure of the CSGH product generated from CSH and dicyandiamide was confirmed by FTIR. As can be seen in Figure 2, the broad band at 3437 cm^{-1} in the spectrum of chitosan corresponded to the combination of O—H and N—H stretching vibrations, which moved to lower frequency in the spectrum of CSGH. Because of the large amounts of intermolecular and intramolecular hydrogen bonding and the variety of the length and strength of hydrogen bonding, the stretching vibration band appeared in a wide frequency range. Peaks at 1634 and 1522 cm^{-1} were assigned to the stretching vibration of C═N and the distortion vibration of N—H, respectively. The peak at 1314 cm^{-1} was assigned to the stretching vibration of C—N,

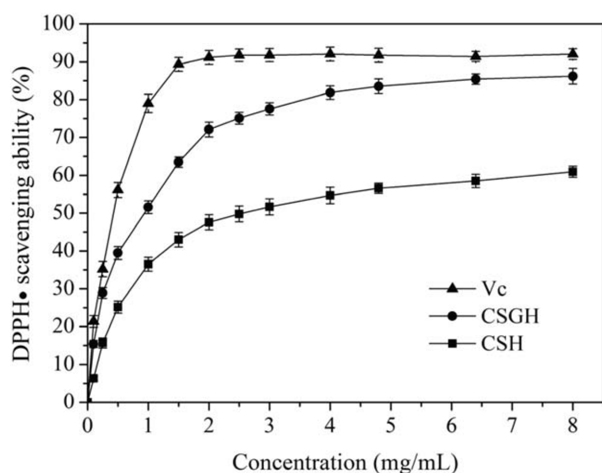

Figure 3. DPPH• scavenging ability of Vc, CSH, and CSGH. Data are presented as means ± SD, $n = 3$.

and the new stronger peak at 1069 cm^{-1} could be associated with the stretching vibration of C—N—C. The peaks at 1634, 1522, and 1378 cm^{-1} in the CSGH spectrum indicated the successful guanidinylation on chitosan.[28]

Determination of Substitution Degree

The substitution degree (DS) of CSGH according to potentiometric titration was 47.2 ± 0.1% (parallel $n = 3$). Element analysis showed that the C/N ratio was 2.097 ± 0.005 (parallel $n = 3$). According to eq. (4), the DS of CSGH was 47.4 ± 0.2%, which was consistent with the result of potentiometric titration.

Molecular Weights

Because of the important role of molecular weight in the bioactivities of chitosan and its derivatives, the M_w of CSH was measured before and after microwave reaction with dicyandiamide, as well as in a blank experiment where CSH solution was treated at the same microwave conditions without dicyandiamide. The GPC results showed that the M_w of CSH was 4.4×10^4. After microwave irradiation was applied to the CSH solution, the M_w of the microwaved CSH decreased to 3.1×10^4. The M_w of the CSGH product was 4.2×10^4. The decrease of the M_w of CSH was the result of degradation of the polymer backbone during the dissolution process of raw chitosan in HCl. The microwave treatment caused a significant degradation of chain length, as reflected in the lowered M_w of the microwaved CSH. However, after reaction with dicyandiamide, the M_w of the CSGH product increased to 4.2×10^4. Therefore, during the microwave reaction, the combined actions of the degradation of CSH and the introduction of biguanidine groups made the M_w of CSGH not suffer a significant decrease, as Zhao *et al.* reported.[23] The two-step preparation method in the present study, in which CSH was first prepared and then reacted with dicyandiamide to obtain CSGH, was thus proved to be effective in avoiding too much reduction of the molecular weight of CSGH.

DPPH• Radical Scavenging Ability

The level of free radicals may suffer an unexpected increase for a variety of reasons, such as an exposure to external oxidant substances or a failure in the defense mechanisns.[29] Excess free radicals, especially for ROS, can attack almost all the biomolecules and cause a vicious chain of damage.[2,30] Radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems,[31] thus becoming a common method to evaluate the activity of an antioxidant.[32]

As shown in Figure 3, the DPPH• scavenging abilities of Vc, CSH, and CSGH were all dose-dependent. CSGH performed much better than GSH within the tested concentration range. At 8.0 mg/mL, the DPPH• scavenging abilities of Vc, CSGH, and CSH were 92.1%, 86.2%, and 61.0%, respectively. The EC50 values of Vc, CSGH, and CSH were approximately 0.42, 0.93, and 2.65 mg/mL.

Because of the strong reduction, Vc exhibited a great DPPH• scavenging capacity, which is in accordance with the previous reports.[33,34] The DPPH• scavenging activity of CSH could be attributed to the combination of its amino and the single electron in the DPPH• solution, which made the DPPH• radical paired, thus decreasing the absorbance of the reaction system. CSGH further enhanced the radical scavenging ability due to the introduction of guanidine groups, and the DPPH• scavenging ability of CSGH was close to that of Vc at 8.0 mg/mL.

Effects on Oxidatively Damaged Cells

Normally, the balance between oxidation and antioxidation *in vivo* was maintained by the radical scavenging effect of enzymatic antioxidant, like superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and nonenzymatic antioxidant, like tocopherol and ascorbic acid.[35] An imbalance between oxidation and antioxidation may damage the membrane and biological structures[36] and thus may cause a series of injuries to cell morphology and enzymatic function and expose the organism to dangers.[37–39]

The free-radical scavenging assay estimates the antioxidant abilities mainly through the evaluation of the direct combining ability of chemical substances with free radicals. In order to better simulate the environment *in vivo*, a cell-based assay was employed as a more suitable and representative method to evaluate the antioxidant capacity of CSGH *in vitro*. On the basis of the oxidatively damaged model created with H_2O_2, the viability and oxidative stress indices of HepG2 cells were measured before and after the interference of CSGH or Vc.

Effects on Cell Viability. CSH, CSGH, and Vc were all demonstrated to be nontoxic to the normal HepG2 cells because no dose of them decreased the OD value of the normal cells (shown in Table I). The OD value of the damaged cells with H_2O_2 treatment (model group, 0.3249 ± 0.0463) was apparently lower than that of the normal group (0.5579 ± 0.0725). Compared to the model group (Group D), the OD value increased with the interference of Vc, CSH, or CSGH. Among them, the effect of Vc was kind of slight, and the effect of CSGH was the most obvious under the same concentration. From the perspective of cell viability, the middle dose of Vc, CSH, and CSGH possessed better activity than other doses of them. Markedly, the OD value of Group CSGH-M was 0.5271 ± 0.0245, equivalent to 94.5% of the normal group (Group N).

Effects on Indexes of Oxidative Damage. SOD. SOD is the first enzyme that responds to oxygen radicals and can scavenge

Table I. Effects of Vc and CSGH on the Activity of Normal or Oxidatively Damaged HepG2 cells

Group	Dose (μg/mL)	OD value of normal cells	OD value of damaged cells
Group N	—	0.5579 ± 0.0725	—
Group D	—	—	0.3249 ± 0.0463
Group Vc	60	0.5803 ± 0.0639	0.3935 ± 0.0087[a]
	300	0.5765 ± 0.0491	0.4118 ± 0.0163[a]
	600	0.5784 ± 0.0638	0.4086 ± 0.0459[a]
Group CSH	60	0.5645 ± 0.0223	0.4295 ± 0.0304[a]
	300	0.5745 ± 0.0151	0.4767 ± 0.0217[a]
	600	0.5796 ± 0.0294	0.4164 ± 0.0288[a]
Group CSGH	60	0.5589 ± 0.0303	0.4842 ± 0.0163[a]
	300	0.5680 ± 0.0259	0.5271 ± 0.0245[a]
	600	0.5788 ± 0.0439	0.5018 ± 0.0204[a]

Data are presented as the mean ± SD; $n = 8$ in each group.
[a] $p < 0.05$ versus group D.

the superoxide anion radicals to protect cells from oxidative damage.[40] Figure 4 shows the effect of CSGH, CSH, and Vc on the SOD activity of oxidatively damaged cells. All three doses of CSH showed about the same effect on increasing the SOD activity. Obviously, CSGH could significantly enhance the SOD activity, and this effect of recovery was not dose-dependent. Vc could improve the SOD activity, but the effect of Vc was smaller than CSGH under the same dose.

MDA. The level of MDA, one of the end products of peroxidation[41] of oxidatively damaged cells, was detected before and after interference with CSGH, CSH, or Vc. As exhibited in Figure 5, setting the model group as control, all doses of CSGH showed a certain ability to decrease the MDA level of oxidatively damaged HepG2 cells. At the same dose, the effect of CSGH on reducing the MDA level was obviously better than CSH. Vc-M slightly decreased while Vc-L and Vc-H increased the MDA level.

GSH-Px and Cell Morphology. GSH-Px is a main antioxidant enzyme that scavenges both exogenous and endogenous ROS.[42] As is shown in Figure 6(A), after interference with CSGH, CSH, or Vc for 24 h, the GSH-Px activity of damaged cells was evidently enhanced compared to the untreated ones in Group D. Cells treated with CSGH expressed higher GSH-Px activity in comparison to those treated with Vc or CSH at the same dose. Significantly, damaged cells treated with CSGH-M and CSGH-H showed GSH-Px activity values approximate to the normal cells in Group N, indicating a superior ability of CSGH to impair the oxidative damage and recover the antioxidant function of damaged cells.

As shown in Figure 6(B), the adherent normal cells grew plump in the dense cytoplasm and compactly connected to each other (Group N). After exposure to 50 μM of H_2O_2 for 2 h, the cells contracted and the karyon was blurred, along with less cytoplasm (Group D). In spite of the increased cell size and clearer connecting border, some mutant spindle cells appeared unexpectedly in Group Vc, while after incubation with CSGH or CSH the morphology of the damaged cells recovered well,

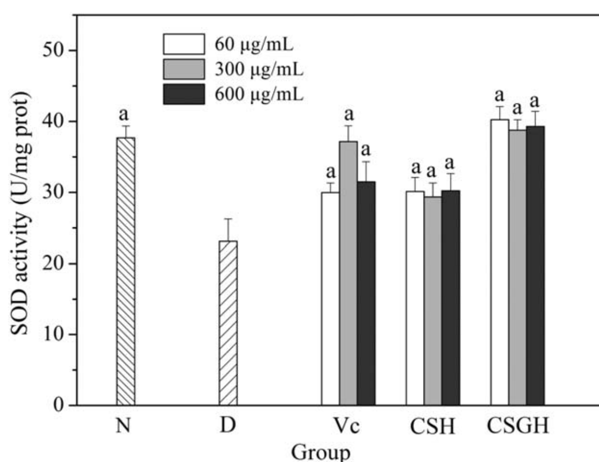

Figure 4. Effects on the SOD activity of oxidatively damaged cells. Data are presented as the mean ± SD, and $n = 8$ in each group; (a) $p < 0.05$ versus group D.

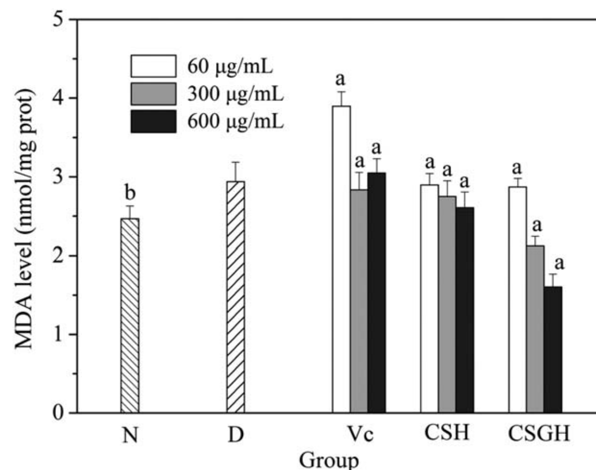

Figure 5. Effects on the MDA level of oxidatively damaged cells. Data are presented as the mean ± SD, and $n = 8$ in each group: (a) $p < 0.05$ versus group D; (b) $p < 0.01$ versus group D.

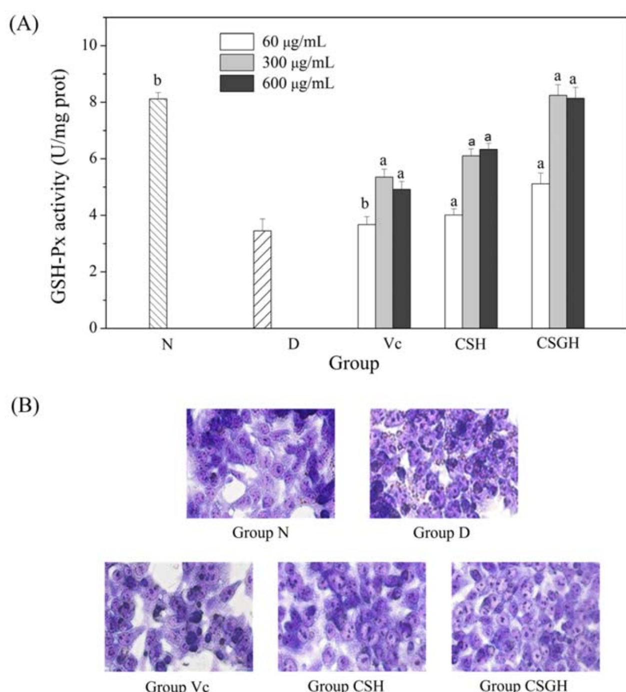

Figure 6. (A) Effects on the GSH-Px activity of oxidatively damaged cells. Data are presented as the mean ± SD, and $n = 8$ in each group: (a) $p < 0.05$ versus group D; (b) $p < 0.01$ versus group D. (B) Morphologies of the oxidatively damaged cells (magnification: ×200). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

shaped as round with the great soma, equal plasm, and clear nucleolus (shown in Group CSGH and Group CSH). An increase in cell size and cytoplasm, as well as more distinct cell borders compared to Group D, was also observed. CSGH and CSH seemed to be superior to Vc on account of the better growth and denser cytoplasm of the cells, demonstrating a significant effect of CSGH and CSH on restoring the morphology of oxidatively damaged cells.

From the indexes of oxidative damage above, CSGH possessed a superior performance to CSH in increasing cell viability, decreasing the level of MDA of oxidatively damaged cells, and recovering cell morphology, along with a noteworthy enhancement of SOD and GSH-Px activity. Interestingly, Vc did not exhibit a corresponding antioxidant ability as it showed in the free radical scavenging experiment. Conversely, Vc-L and Vc-H even increased the level of MDA, the end product of lipid oxidation, suggesting the possible promotion action of Vc to the oxidation. For a long time Vc has been used as an exogenous antioxidant reagent against oxidative damage in bodies. However, improper use of Vc might increase the sensitivity of DNA in cells to the damage caused by H_2O_2 or other harmful substances.[43] Vc at low doses offered only feeble protection to oxidatively damaged DNA, but high-dose Vc could promote the rupture of DNA chains and thus accelerate the damage.[44] Recent studies also proved that Vc promoted forestomach carcinogenesis[45] and esophageal carcinogenesis.[46]

Based on the analysis and comparison of the results above, we suggested the antioxidant effect of chemical agents such as Vc could be assigned to its bonding ability to free radicals rather than the recovery effect on the organism, and its possible side effects still need more attention. Despite the slightly inferior results in the free radical scavenging experiment, CSGH and CSH overmatched Vc in repairing the form and improving the antioxidant ability of cells and thus impairing the oxidatively damaged cells to a deeper level, remarkably. The introduction of the biguanidine group to chitosan successfully improved the antioxidant ability of chitosan, as reflected in the better impairing effects of CSGH than CSH. Namely, the antioxidant activity of CSGH has mainly to do with its recovery effect on damaged cells rather than simple scavenging action to free radicals, indicating the potential capacity of CSGH for regulating the oxidation/antioxidation balance. Such behavior could not only improve the cell viability but also enhance the activity of antioxidant enzymes.

CONCLUSIONS

Chitosan biguanidine hydrochloride (CSGH) was prepared under microwave irradiation. The antioxidant ability of CSGH was better than that of CSH and Vc. Notably, the intervention of CSGH could help to enhance the activities of antioxidant enzymes of oxidatively damaged cells. CSGH will be a potential repairing antioxidant against oxidative damage and may help to recover the antioxidant function of damaged cells. Further verification *in vivo* remains to be carried out.

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