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Anti-Photoaging effects and safety evaluation of chitosan Maillard derivatives: *in vitro* and *in vivo* studies

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ABSTRACT: Ultraviolet B (UVB) radiation is a major contributing factor to skin photoaging by accelerating inflammation and collagen degradation. This underscores the critical need to develop natural and safe bioactive compounds to combat UVB-induced skin disease. In this study, different chitosan Maillard reaction products (CMRPs) were prepared and characterized to evaluate their anti-photoaging activities, mechanisms of action and safety. The Maillard reaction altered the morphology of chitosan from a loose powder into a crosslinked structure. The resulting CMRPs exhibited enhanced DPPH and ABTS radical scavenging activities as well as increased elastase inhibitory effects. All six CMRPs were non-toxic to HaCaT cells and improved the viability of UVB-induced photoaged cells, with CG-6 demonstrating the most potent anti-photoaging activity. Mechanistic investigations revealed that CG-6 significantly inhibited the accumulation of reactive oxygen species (ROS) in photoaged HaCaT cells, enhanced the activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), suppressed the expression of matrix metalloproteinases (MMPs) and inflammatory factors, and regulated the expression of key genes in the MAPK signaling pathway, thereby exerting protective effects against photoaging. Oral administration of CG-6 for 28 days did not cause any apparent organ damage in mice. There were no significant differences in organ indices, histopathological examination, hematological parameters, liver and kidney function markers, or antioxidant enzyme activity levels between the CG-6-treated and control groups. These findings indicate that CG-6 does not cause negative effect on the health of mice. Overall, this study provides a strong theoretical foundation for the application of Maillard reaction products, particularly CG-6 in the development of anti-photoaging functional foods.

Keywords: Maillard reaction, chitosan, derivatives, anti-photoaging, subacute toxicity.

1. Introduction

The Maillard reaction is a non-enzymatic browning process that occurs between reducing sugars and amino compounds under thermal conditions. It begins with the formation of glycosylamines which undergo Amadori rearrangement followed by a series of dehydration, cleavage, and condensation reactions. These sequential steps ultimately lead to the production Maillard reaction products, which are characterized by their distinctive color, aroma, and diverse biological activities^[1]. Commonly occurring in food processing, the

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Maillard reaction is a key mechanism responsible for the flavor development and browning observed in baked and roasted foods. In recent years, Maillard reaction products have garnered significant attention in food science and biomedicine due to their antioxidant, antimicrobial, anti-inflammatory, and immunomodulatory properties [2].

Chitosan, a natural cationic polysaccharide obtained through the deacetylation of chitin, exhibits excellent biocompatibility, biodegradability, and diverse biological activities. Owing to abundance of amino functional groups, chitosan can act as an amino donor in the Maillard reaction with monosaccharides (e.g., glucose, xylose) leading to the formation chitosan Maillard reaction products [3, 4]. Hereafter, the derivatives obtained from the Maillard reaction between chitosan and monosaccharides are referred to as chitosan–Maillard reaction products (CMRPs). These CMRPs not only preserve the favorable physicochemical properties of chitosan but also demonstrate enhanced bioactivities—such as improved antioxidant and antimicrobial effect—attributed to the incorporation of nitrogen—containing heterocycles and phenolic structures [5, 6]. CMRPs have shown promising application potential owing to their natural origin and simple preparation processes, particularly in the application of functional foods and biomedicine [7]. However, the heterogenous composition of Maillard reaction products and potential safety concerns continue to limit their widespread utilization. Therefore, systematic evaluation of the *in vivo* safety of CMRPs is essential to support their further development and application.

Skin aging is a natural, multifactorial process influenced by both intrinsic and extrinsic factors. Among these, photoaging primarily induced by chronic ultraviolet (UV) radiation exposure, is closely link to various skin disorders, including squamous cell carcinoma, and melanoma [8, 9]. Numerous studies have reported the anti-photoaging effects of dietary peptides [10], polysaccharides [11], and other bioactive compounds [12]. Compared with conventional drugs, these natural compounds are generally milder, safer, and less irritating to the skin. However, the complex purification procedures, high production costs, and variability in structure due to processing and raw material quality have limited their further industrial application [13, 14]. Although CMRPs have been reported to exhibit strong antioxidant activity, their ability to counteract UV-induced oxidative stress and prevent photoaging has not been investigated or reported in the existing literature.

In this study, chitosan was used as a raw material and reacted with glucose or xylose under moist-heat conditions to generate a series of CMRPs with varying degrees of reaction. The structural characteristic of these CMRPs were systemically analysed, and their anti-photoaging potential was assessed using a UVB-induced photoaging model in HaCaT cells. Furthermore, a 28-day oral toxicity evaluation in mice was performed to determine their *in vivo* safety. Collectively, this study provides a theoretical foundation for the development and utilization of CMRPs as functional ingredients in anti-photoaging foods.

2. Materials and methods

2.1. Materials and chemicals

Chitosan (30 kDa) was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). Glucose and xylose were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Commercial kits for SOD,

LDH, GSH-Px, and BCA assays were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), while CAT and MDA kits were purchased from Beyotime Co., Ltd. (Shanghai, China). All other chemicals and reagents were of analytical grade.

2.2 Preparation of chitosan Maillard reaction products

The chitosan Maillard reaction products (CMRPs) were prepared according to the method of Chekanouskaya et al. with some modifications^[15]. Briefly, chitosan was dissolved in acetic acid (1%, v/v), and then an equal volume of (4% (w/v), 5% (w/v), 6% (w/v)) glucose solution and (1% (w/v), 1.5% (w/v), 2% (w/v)) xylose solution was added to the chitosan solution, respectively. The pH value of six mixed solutions was adjusted to 5.5 using 1 mol/L aqueous NaOH. Each mixture was then placed in an autoclave at 121 °C for 30 minutes, then the mixtures were immediately transferred to an ice bath to rapidly cool down to room temperature. Subsequently, the samples were then dialyzed (cut-off of MW of 10 kDa) with deionized water for 48 h. Derivatives obtained from chitosan-glucose reactions at 4%, 5%, and 6% concentrations were designated as CG-4, CG-5, and CG-6 respectively, while those from chitosan-xylose at 1%, 1.5%, and 2% concentration were labeled as CX-1, CX-1.5, and CX-2, respectively.

2.3 Measurement of Ultraviolet–visible absorbance and browning

An Ultraviolet-visible spectrophotometer (UV-2204; Shanghai Spectroscopic Instrument Co., Ltd., Shanghai, China) was used to measure the absorbance at 290 nm and 420 nm (browning intensity) for different chitosan Maillard derivatives.

2.4 Fluorescence spectroscopy analysis of CMRPs

Fluorescence spectra were determined by a fluorescence spectrophotometer (Fluorolog-3, HORIBA, Ltd., Kyoto, Japan), and the parameters were set as follows: excitation wavelength was 320 nm, and the scanning emission spectrum range was 340–550 nm.

2.5 Degree of grafting (DG) measurement

The grafting degree (DG) was determined according to the method of Li et al, with some modifications^[16]. Briefly, 200 µL of chitosan Maillard derivatives were mixed with 1 mL of OPA reagent, using distilled water as a blank. The absorbance was measured at 340 nm and recorded as A_0 . After incubation at 35 °C for 3 min, the absorbance was measured again and recorded as A_1 . The DG of each samples was then calculated using the following formula:

$$DG(\%) = \frac{A_0 - A_1}{A_0} \times 100\%$$

2.6 FT-IR Spectra Analysis

The Fourier transform infrared (FTIR) spectra of chitosan Maillard derivatives were recorded with a FTIR spectrophotometer (Tensor 27, Bruker Co. Ltd., Bergisch Gladbach, Germany) from 4000 cm⁻¹ to 500 cm⁻¹.

2.7 SEM Analysis of CMRPs

The morphology and microstructure of CMRPs were examined using SEM (EVO 18, Carl Zeiss AG, Oberkochen, Germany). Lyophilized polysaccharides were sputter-coated with a thin gold layer, mounted on the microscope stage, and imaged under vacuum at an accelerating voltage of 10 kV. Micrographs were acquired at magnifications of 100× and 500×.

2.8 Antioxidant Activity of CMRPs

2.8.1 DPPH Radical Scavenging Activity

Sample solutions (1 mL) at varying concentrations were mixed with 1 mL of DPPH (150 μ mol/L in ethanol) and incubated in the dark for 30 min. Absorbance was recorded at 517 nm using a multifunctional microplate reader (SpectraMAX250, Molecular Devices, Sunnyvale, CA, USA). The control contained only DPPH and ethanol, while a sample background (sample and ethanol without DPPH) was prepared in parallel. Results were expressed as IC₅₀, defined as the sample concentration required to scavenge 50% of DPPH radicals.

2.8.2 ABTS Radical Scavenging Activity

The ABTS radical scavenging activity was determined to assess the antioxidant activities of CMRPs. In brief, 20 μ L of sample solution at different concentrations was added to a 96-well plate and mixed with 180 μ L of ABTS⁺ working solution (prepared by diluting the ABTS radical cation with ethanol to an absorbance of 0.6–0.9). Distilled water served as the blank control. After thorough mixing, absorbance was recorded at 734 nm using a microplate reader (Molecular Devices, USA). Results were expressed as IC₅₀, defined as the sample concentration required to scavenge 50% of ABTS radicals.

2.8.3 Elastase Inhibition Activity Assay

Elastase inhibitory activity was assessed following a previously reported method with some modifications^[10]. Reactions were performed in 100 mmol/L Tris-HCl buffer (pH 8.0). Test samples (20 μ L) were mixed with 100 μ L of elastase solution (100 U/mL) and incubated at 37 °C for 20 min. Subsequently, 80 μ L of the substrate N-(methoxysuccinyl)-Ala-Ala-Pro-Val p-nitroanilide (2 mmol/L) was added and incubated for another 20 min at 37 °C. Absorbance was then measured at 410 nm using a microplate reader (SpectraMAX250, Molecular Devices, CA, USA). Elastase inhibition activity was calculated according to Equation (1), where OD_{control} and OD_{sample} corresponded to the optical density of the control and sample groups, respectively.

$$\text{Elastase inhibition (\%)} = \left(\frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \right) \times 100\% \quad (1)$$

2.9 Anti-photoaging activity of CMRPs in vitro

2.9.1 Cell culture and UVB irradiation

The human immortalized keratinocyte cell line HaCaT was obtained from the Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China). HaCaT cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. Prior to UVB irradiation,

cells were washed with PBS and maintained in PBS-filled wells, then exposed to UVB (15 mJ/cm²) using a UVB lamp (UVB-313 nm, 8 W; Shenzhen Guanhongrui Instrument Co., Ltd., Shenzhen, China). After irradiation, PBS was removed and fresh DMEM containing 10% FBS and 1% penicillin–streptomycin was added, followed by incubation at 37 °C with 5% CO₂.

2.9.2 Evaluation of HaCaT Cell Viability

HaCaT cells (1.0×10^4 cells/well) were seeded in 96-well plates and treated with chitosan Maillard derivatives in serum-free medium for 12 h. Sample concentrations were chosen based on prior experimental experience. After UVB exposure, cells were further incubated for 12 h in DMEM containing 10% FBS and 1% penicillin–streptomycin. All groups were subjected to UVB irradiation except the control. Cell viability was assessed using the MTT assay following the manufacturer's instructions.

2.9.3 Determination of ROS content in HaCaT cells

HaCaT cells (5×10^6 cells/well) were seeded in 6-well plates and incubated for 24 h at 37 °C, followed by treatment with CMRPs for 12 h, with hyaluronic acid (HA) serving as the positive control. After treatment, the medium was removed and cells were washed with PBS. DCFH-DA ($10 \mu\text{mol/L}$ in serum-free DMEM) was then added and incubated for 30 min at 37 °C, followed by another PBS wash. Finally, cells were maintained in serum-free DMEM, and fluorescence signals were measured using a microplate fluorescence reader (Molecular Devices, USA) at 37 °C.

2.9.4 Determination of the activities of SOD and CAT in HaCaT cells

HaCaT cells (1.5×10^7 /well) were cultured for 24 h, washed with PBS, trypsinized with trypsin-EDTA, and reseeded into 6-cm dishes at 1.5×10^6 cells per dish. Cells were pretreated for 12 h with chitosan Maillard derivatives (200, 300, or 400 $\mu\text{g/mL}$) or HA (400 $\mu\text{g/mL}$) and then treated as described in Section 2.9.2. Following UVB irradiation, cells were incubated for an additional 12 h, washed with PBS, lysed, and centrifuged (10,000 rpm, 10 min) to obtain the supernatants. The activities of SOD and CAT, along with GSH levels, were determined using commercial assay kits, and protein content was quantified with a total protein assay kit.

2.9.5 Determination of the contents of MMP-1, MMP-3, and IL-6

The effects of CMRPs on the level of MMP-1, MMP-3, and IL-6 in photoaged HaCaT cells were evaluated using enzyme linked immunosorbent assay (ELISA). HaCaT cells were incubated in 6-cm culture dishes at a concentration of 1.5×10^6 cells per dish and treated following section 2.9.4. Following treatment, the cells were lysed, and centrifuged to collect the supernatants. The ELISA kit was used to measure the contents of MMP-1, MMP-3, IL-6, and all values were normalized against total protein measured by the BCA assay. HA was used as a positive control.

2.9.6 Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from HaCaT cells using Trizol (Thermo-Fisher, Grand Island, NY, USA), and cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo-Fisher, Grand Island, NY,

USA), following the manufacturer's instructions. qRT-PCR was then performed using SYBR Green Master qPCR Mix (TSINGKE, Beijing, China), and the primers (Sangon Biotech, Shanghai, China) were shown in Table 1, which were designed by Oligo 7 software (Molecular Biology Insight, Inc., Cascade, CO, USA). Changes in gene expression were analyzed using the CFX48™ Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and fold changes were calculated using $2^{-\Delta\Delta CT}$ method, normalized with GADPH as the housekeeping gene.

Table 1 Primers sequences of related genes

Gene		Primers sequences (5'-3')
<i>Gapdh</i>	Forward	TCCACTGGCGTCTTCACCACCAT
	Reverse	GGAGGCATTGCTGATGATCTTGAGG
<i>Mmp-1</i>	Forward	GATGTGGAGTGCCTGATGTG
	Reverse	TGCTTGACCCTCAGAGACCT
<i>Mmp-3</i>	Forward	CACTCACAGACCTGACTCGG
	Reverse	GAGTCAGGGGGAGGTCCATA
<i>Mmp-9</i>	Forward	AGTCCACCCTTGTGCTCTTCC
	Reverse	TGCCACCCGAGTGTAAACCAT
<i>Erk</i>	Forward	GCCGAAGCACCATTCAAGTT
	Reverse	CCTCTGAGCCCTTGTCTCTGA
<i>Jnk</i>	Forward	CAAGGTGTTGTGCGATGACG
	Reverse	TGGTTTCTTGGTCGGTGGG
<i>p38</i>	Forward	CTGTACCTGTCCTGCGTGTT
	Reverse	AGACGGGCATGTTTTCTGCT
<i>Tnf-α</i>	Forward	GCTGCACTTTGGAGTGATCG
	Reverse	CTTGTCACCTCGGGGTTTCGAG
<i>Il-6</i>	Forward	CTGACCCAACCACAAATGC
	Reverse	TCTGAGGTGCCCATGCTAC
<i>Nf-κb</i>	Forward	ACAGATGGCCCATACTTCAAA
	Reverse	ATAACCTTTGCTGGTCCCACAT
<i>p53</i>	Forward	GTGACACGCTTCCCTGGATTG
	Reverse	ATTCTGGGAGCTTCATCTGGAC

2.10 Animals and treatment

Eighty healthy Balb/c mice (4 weeks old, weight 20 ± 2 g) were purchased from ZhuHai Bestest Biotechnology Co., Ltd. (Zhuhai, China) (Certificate number SCXK (Yue)-2020-0051). In the current study, the experimental period, grouping, and dose setting were determined according to the National Standard of the People's Republic of China GB15193.22-2014. All the experimental mice were adaptively fed for one week before the experiment, and then a 28-day experiment was conducted. A control group, low-dose, medium-dose and high-dose groups were set up. Forty male and forty female mice were randomly divided into 10 groups. Each group was named CB (male control group), LB (male low-dose group, 1.0 g/kg bw/day), MB (male medium-dose group, 2.0 g/kg bw/day), HB (male high-dose group, 3.0 g/kg bw/day), CG (female control group), LG (female low-dose group, 1.0 g/kg bw/day), MG (female medium-dose group, 2.0 g/kg bw/day), and HG (female high-dose group, 3.0 g/kg bw/day), respectively. All mice were fed with normal feed. The low-dose, medium-dose and high-dose groups were gavaged with CMRPs named CG-6, while the control group was gavaged with distilled water. The daily body weight of the mice was recorded and the sample dosage was calculated based on the daily body weight, that for the MB and MG groups was 2.0 g/kg

bw/day, that for the HB and HG groups was 3.0 g/kg bw/day, and mice in the CB and CG groups were gavaged with the same volume of distilled water. After 28 days of experiments, the blood samples were collected from the eyeballs of mice, and then the mice were sacrificed. Before usage, all organs and excrement were kept at -80 °C.

2.11 Evaluation of subacute toxicity of CMRPs CG-6

2.11.1 Histopathologic analysis

Fresh liver, spleen, and kidney tissues were fixed in 10% formaldehyde, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E) for histological examination under an Olympus BX43 microscope (Olympus, Tokyo, Japan). Frozen sections (5 µm) were stained with Oil Red O (Nakarai Tesque Inc., Kyoto, Japan) and counterstained with hematoxylin.

2.11.2 Determination of serum indexes

Blood samples were centrifuged at 3,000 rpm for 10 min to obtain serum. Routine blood parameters were measured using an automated biochemical analyzer (Model 3100, Hitachi, Tokyo, Japan).

2.11.3 Determination of liver biochemistry

The levels of CAT, SOD, MDA, GSH-Px and LDH in mice livers were measured using kits following the manufacturer's instructions. The total protein content was used to normalize all of the data.

2.12 Statistical analysis

The data were analyzed using SPSS 26 software (IBM, USA) by one-way ANOVA, and differences between groups were evaluated with Tukey's test at a 95% confidence level ($P < 0.05$). The significant difference between groups was examined with Tukey's tests for means with 95% confidence limit ($P < 0.05$). Data were presented as the mean $\pm$ standard deviation.

3. Results and discussion

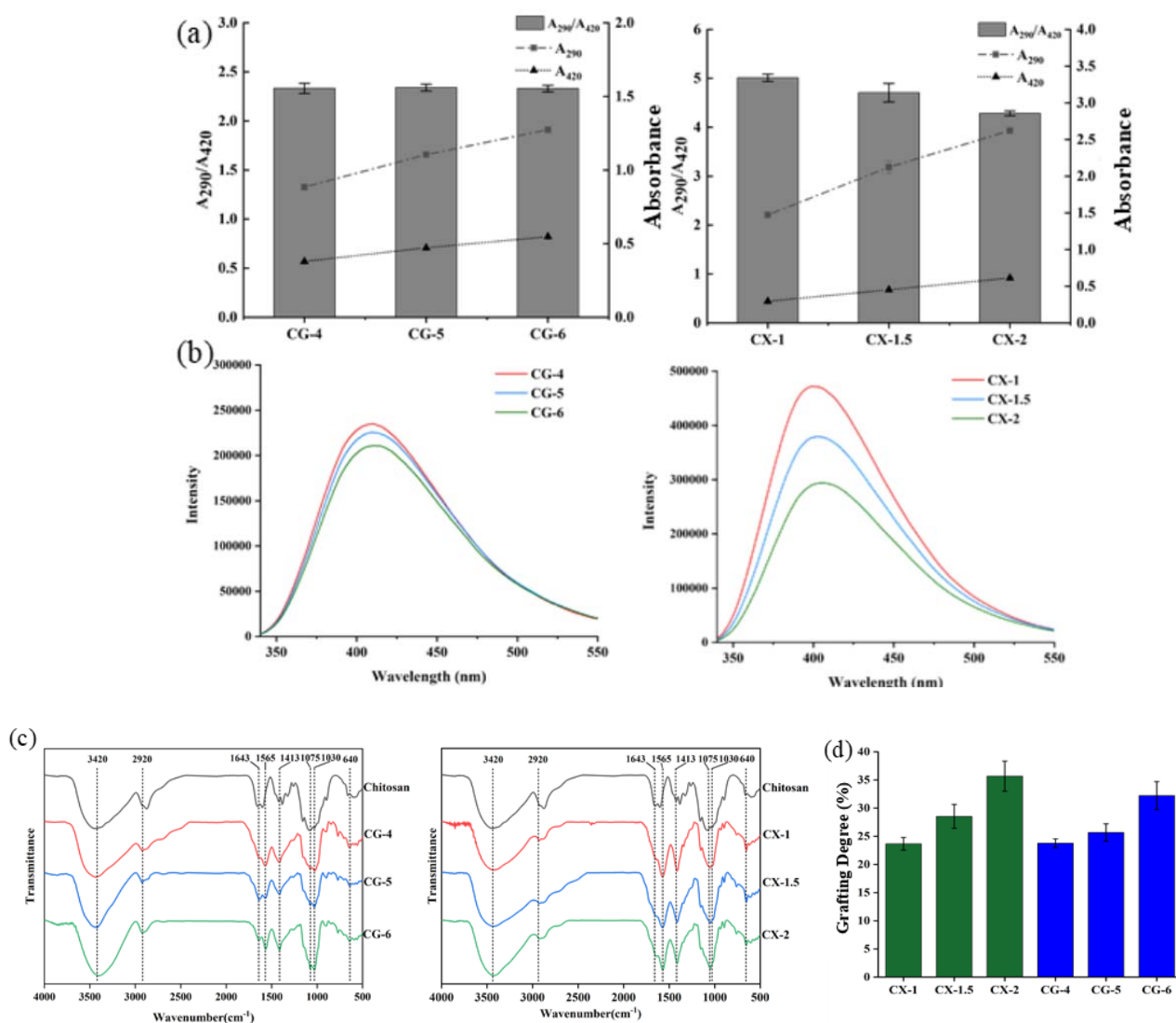
3.1 Structure characterization of different CMRPs

3.1.1 Ultraviolet absorption analysis

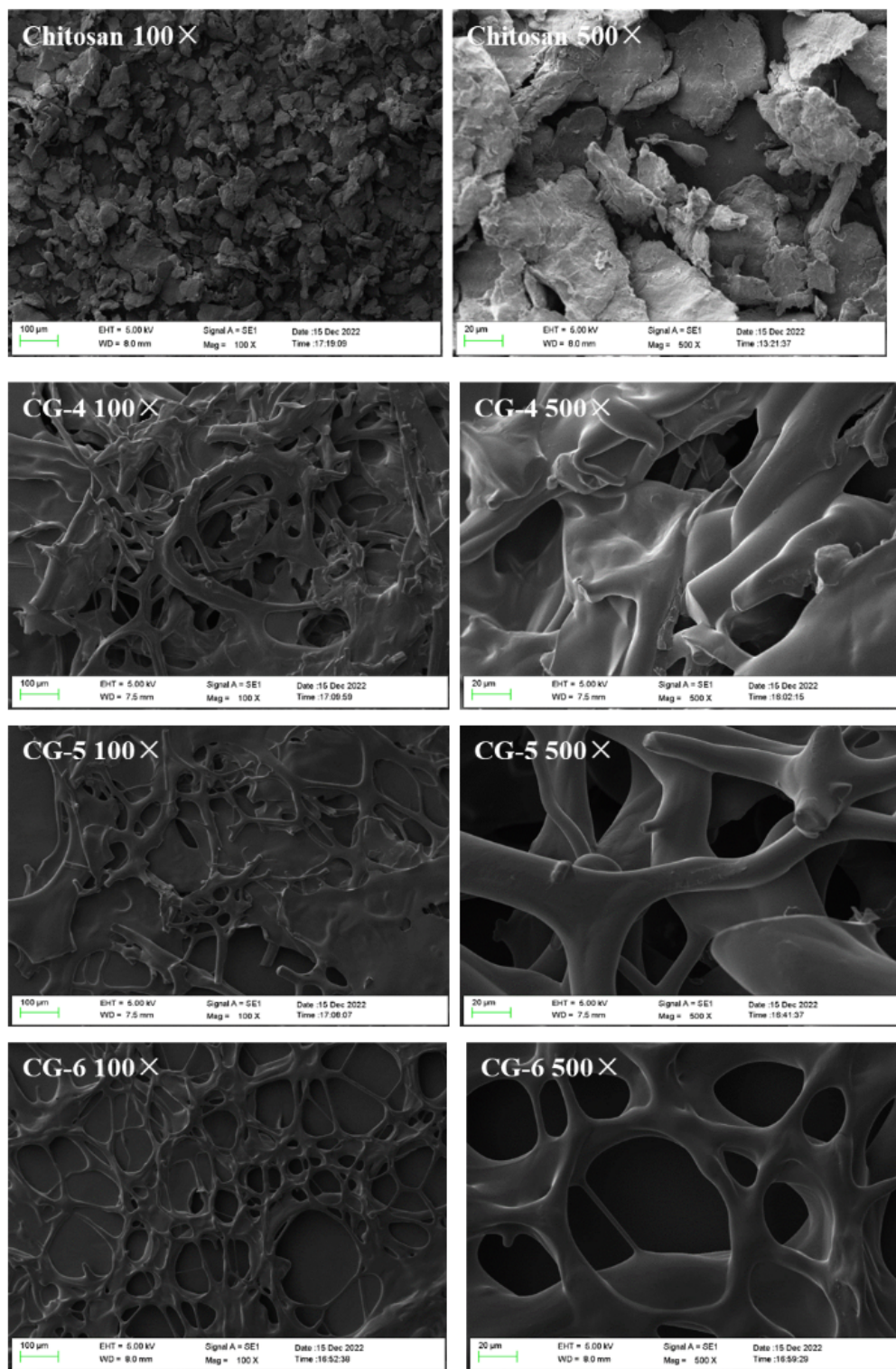
The Maillard reaction can be divided into three stages based on the formation of reaction products: the initial, intermediate and final. In the initial and intermediate stages, colorless Amadori rearrangement products are formed through non-enzymatic reactions between the aldehyde carbonyl or ketone carbonyl of sugars and amino acids or other free amino compounds. The absorption of these reaction intermediates can be detected at a wavelength of 290 nm. As the reaction proceeds to the final stage, a series of complex reactions produce brown polymers known as melanoidins, which show characteristic absorption at 420 nm. The intensity of absorption at 420 nm serves as an indicator of browning and the extent of the Maillard reaction^[17].

As shown in Figure 1a, the absorbance of the derivatives at both 290 nm and 420 nm increased with rising concentrations of glucose or xylose in the reaction. When the glucose concentration increased from 4% to 6%, the absorbance of the CMRPs rose from 0.88 to 1.27 at 290 nm, and from 0.38 to 0.55 at 420 nm.

Similarly, when the xylose concentration increased from 1% to 2%, the absorbance at 290 nm increased from 1.47 to 2.62, and at 420 nm from 0.29 to 0.61. These results indicate that higher monosaccharide concentration leads to a more intense Maillard reaction, with greater accumulation of intermediate and late-stage products, which is reflected in the observed browning of the reaction products. The chitosan-xylose reaction generated significantly more products with absorbance at 290 nm than the chitosan-glucose reaction. Furthermore, the absorbance at 420 nm of the product formed by the reaction of 2% xylose with chitosan (CX-2) was also higher than that of the product formed by the reaction of 6% glucose with chitosan (CG-6). Although the xylose concentration used in the reaction was lower than glucose, the reaction with xylose resulted in more Maillard intermediates and late-stage products were obtained. This differences may be attributed to the reactivity of different sugars, as steric hindrance and molecular weight can affect the reaction rate [18]. According to the previous study, pentoses react faster than hexoses, and monosaccharides react faster than disaccharides or oligosaccharides [19].



(e)



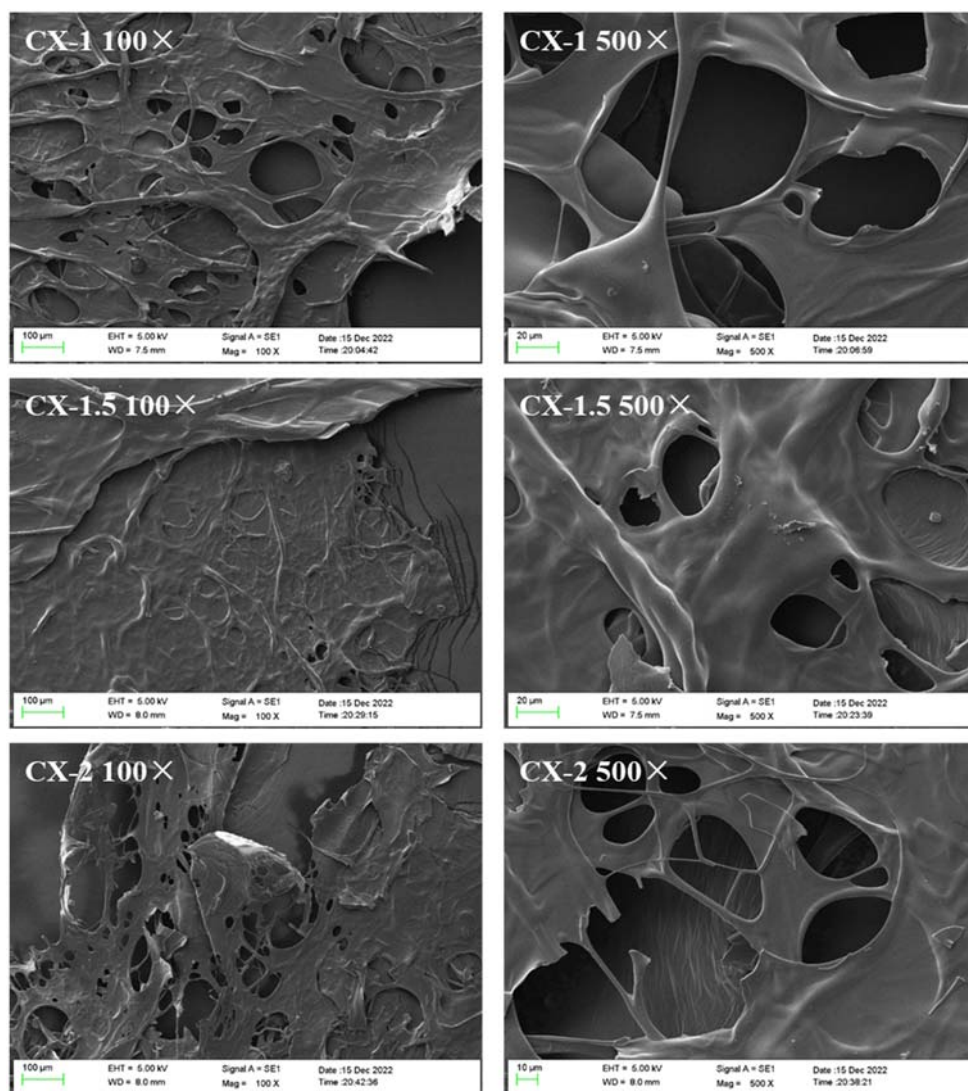


Figure 1 Chemical structure of CMRPs. (a) absorbance of chitosan-glucose Maillard derivatives (left) and chitosan-xylose Maillard derivatives (right) at 290 nm and 420 nm; (b) fluorescence spectrum of different CMRPs; (c) FT-IR spectrum of different CMRPs; (d) grafting degree of different CMRPs; (e) scanning electron micrograph of chitosan and CMRPs at different multiples.

As the reaction proceeds to the late stages, intermediate products are converted into late-stage products, resulting in a decrease in the A_{290}/A_{420} ratio^[20]. The A_{290}/A_{420} values of the chitosan-glucose Maillard derivatives CG-4, CG-5, and CG-6 were 2.33, 2.34, and 2.33, respectively, showing no significant changes. In contrast, the A_{290}/A_{420} values of the chitosan-xylose reaction products CX-1, CX-1.5, and CX-2 were 5.01, 4.70, and 4.28, respectively, exhibiting a gradual decreasing trend. This decrease indicates that the reaction has entered the late stage. In summary, with increasing monosaccharide concentration, more Maillard reaction intermediates and late-stage products accumulate, with the chitosan-xylose reaction producing more intermediates than the chitosan-glucose reaction. Furthermore, the A_{290}/A_{420} values of the chitosan-glucose reaction products remained stable, while those of the chitosan-xylose reaction products continuously decreased. This suggests that the chitosan-xylose reaction proceeds more rapidly and enters the late stage sooner than the chitosan-glucose reaction.

3.1.2 Fluorescence spectral analysis

In the intermediate stage of the Maillard reaction, fluorescent intermediates are formed, which are considered precursors of melanoidins. As the reaction proceeds to the late stage, these intermediates are converted into non-fluorescent melanoidins, resulting in a decrease in fluorescence intensity. Figure 1b showed that as the glucose concentration increased from 4% to 6%, the fluorescence intensity of the derivatives slightly declined, from 2.35×10^6 to 2.11×10^6 . In contrast, when the xylose concentration increased from 1% to 2%, the fluorescence intensity of the derivatives significantly decreased, from 4.59×10^6 to 2.92×10^6 . A similar trend was observed in the previous study, where the fluorescence intensity of reaction products initially increased and then decreased [21]. Additionally, the fluorescence intensity of the chitosan-xylose Maillard derivatives (CX-1, CX-1.5, CX-2) was higher than that of the chitosan-glucose derivatives (CG-4, CG-5, CG-6), indicating greater accumulation of intermediates, which was consistent with the absorbance trends at 290 nm described in Section 3.1.1. These results demonstrate that increasing monosaccharide concentration accelerates the Maillard reaction process, promoting the conversion of intermediates into late-stage melanoidins. Additionally, the reaction between chitosan and xylose proceeds more rapidly than the reaction with glucose.

3.1.3 Grafting degree

During the Maillard reaction, the free amino groups in chitosan react with the carbonyl groups of sugars and are consequently consumed [22]. The grafting degree of the Maillard reaction products can be calculated by measuring the content of free amino acids remaining in the final products. A lower residual free amino group content indicate a higher degree of grafting, suggesting a more complete reaction.

As shown in Figure 1d, the degree of grafting of the six CMRPs increased with rising xylose concentration from 1% to 2%, as more amino groups were consumed, with the grafting degree increasing from 23.67% to 35.67%. A similar trend was observed in the chitosan-glucose reaction, where the grafting degree of the CMRPs increased from 23.77% to 32.22% as the glucose concentration rose from 4% to 6%. The degree of grafting of the CMRPs followed the order: CX-2 > CG-6 > CX-1.5 > CG-5 > CG-4 > CX-1, which was consistent with the absorbance trends at 290 nm and 420 nm described in Section 3.1.1. These results suggest that increasing monosaccharide concentration accelerates the Maillard reaction, leading to a greater accumulation of CMRPs.

3.1.4 Infrared spectral analysis

The functional group changes of substances can be analyzed using infrared (IR) spectroscopy. The IR spectra of chitosan and its six CMRPs were shown in Figure 1c, with the scanning range of 500–4000 cm^{-1} . Compared with chitosan, the Maillard derivatives exhibited varying degrees of shifts and changes in the positions and intensities of absorption peaks, mainly in the 800–2000 cm^{-1} region.

For both chitosan and its six CMRPs, absorption peaks were observed at 3400 cm^{-1} and 3420 cm^{-1} , corresponding to the stretching vibrations of O-H and N-H groups. Absorption peaks at 2875 cm^{-1} and 2927 cm^{-1} were attributed to the stretching vibrations of C-H bonds, which were characteristic peaks of

polysaccharides^[23]. In the spectrum of chitosan, absorption peaks at 1647 cm⁻¹ and 1602 cm⁻¹ correspond to the amide I and amide II regions, related to the bending vibrations of -NH groups. In the Maillard derivatives, these peaks shifted to 1643 cm⁻¹ and 1565 cm⁻¹, respectively, indicating that the reducing ends of glucose and xylose reacted with the amino groups of chitosan to form Schiff bases with C=N double bonds^[24]. The peak at 1421 cm⁻¹ in the chitosan spectrum is attributed to the bending vibration of -NH₂, while the peak at 1384 cm⁻¹ is attributed to the bending vibration of C-H bonds^[21]. After reacting with glucose or xylose, these two peaks in the chitosan Maillard derivatives shifted to 1413 cm⁻¹, suggesting the consumption of -NH₂ groups. Additionally, the increased intensity of these absorption peaks in the derivatives can be partly attributed to the overlapping of the two peaks and to the introduction of sugar chains into the chitosan molecule through the Maillard reaction, leading to an increase in -CH₂ groups^[25]. The absorption peak at 1158 cm⁻¹ in the chitosan spectrum corresponds to the stretching vibration of C-O bonds, which shifted to 1075 cm⁻¹ after reaction with glucose or xylose. Similar shifts were observed by Umemura et al^[26], who reported that when mannan reacted with chitosan in a Maillard reaction, the phenomenon was attributed to the cleavage of sugar units in chitosan. The stretching and bending vibrations of C-N bonds in chitosan were observed at 1074 cm⁻¹ and 603 cm⁻¹, respectively, corresponding to the amide III region. After reaction with glucose or xylose, these peaks shifted to 1030 cm⁻¹ and 640 cm⁻¹^[27], respectively. These observed shifts of functional groups across the six CMRPs confirm that chitosan undergoes a Maillard reaction with glucose and xylose, leading to the formation of characteristic Maillard products.

3.1.5 Apparent morphology

The structures of chitosan and its Maillard derivatives observed under scanning electron microscopy (SEM) were shown in Figure 1e, with magnifications of 100× and 500×. The images revealed significant differences in the apparent morphology between chitosan and its Maillard derivatives. Introducing carbonyl compounds such as reducing sugars or amino compounds for crosslinking through the Maillard reaction is a common method to improve the properties of chitosan and alter its morphology. The chitosan appeared as fragmented, flaky powder under the microscope. After the Maillard reaction, the six derivatives exhibited porous, crosslinked, network-like structures. Qu et al. also observed that products obtained by ultrasound-assisted moist heat treatment of rapeseed protein-dextran systems exhibited looser and more porous surface structures^[28]. As the concentrations of glucose or xylose increased, the derivatives showed more compact structures. Moreover, the crosslinked structures formed by the Maillard reaction between chitosan and xylose displayed tighter interlayer connections compared to those formed with glucose. These morphological changes indicate that chitosan and glucose or xylose undergoes a chemical reaction, rather than a simple physical mixture.

3.2 Anti-photoaging activities of different CMRPs in vitro

3.2.1 The antioxidant activities of different CMRPs

Figure 2a showed the DPPH radical scavenging activity of the different CMRPs. When the concentration of the glucose increased from 4% to 6%, the DPPH radical scavenging rate of the CMRPs at the same

concentration increased from 54.45% to 68.77%, showing a gradual upward trend. Similarly, when the concentration of the xylose solution increased from 1% to 2%, the DPPH radical scavenging rate increased from 52.77% to 77.16%, exhibiting a gradual upward trend. Figure 2b showed the ABTS radical scavenging activity of the different CMRPs. The figure indicated that as the glucose concentration increased, the ABTS radical scavenging rate of the CMRPs at the same concentration rose from 53.71% to 70.41%, showing a gradual increase. When the xylose concentration increased, the ABTS radical scavenging rate increased from 53.83% to 73.96%, also displaying a gradual upward trend. These results demonstrate that increasing the monosaccharide concentration enhances the DPPH and ABTS radical scavenging rates of the Maillard derivatives.

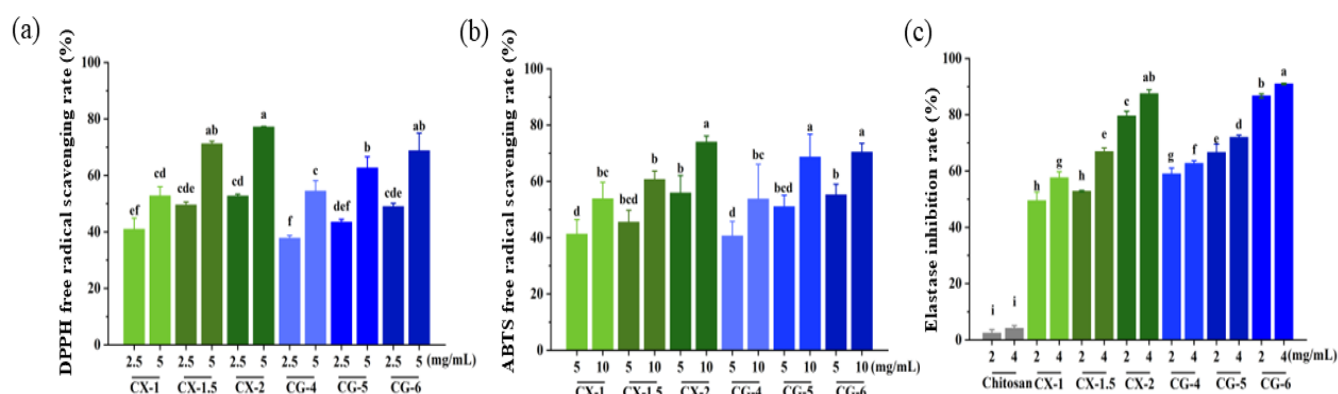


Figure 2 Antioxidant and anti-photoaging activities of different CMRPs. (a) DPPH free radical scavenging rate; (b) ABTS free radical scavenging rate; (c) Elastase inhibition activity of different CMRPs

^{a-d} Values with different letters are significantly different ($P < 0.05$).

The results suggest that higher monosaccharide concentrations promote the Maillard reaction and facilitate the accumulation of reaction products such as reductones and melanoidins, which can exert antioxidant effects by scavenging oxygen radicals^[29], terminating radical chain reactions, or chelating metal ions^[20]. Additionally, although the increase in xylose concentration was smaller than that of glucose, the improvement in radical scavenging rates for the xylose-derived products was greater than for the glucose-derived ones. This indicates that the Maillard reaction between chitosan and xylose proceeds more rapidly, resulting in a faster accumulation of reaction products, which aligns with the trends of increased absorbance and browning intensity described in Section 3.1.

3.2.2 The elastase inhibition rate of different CMRPs

Elastin and collagen are major components of the skin, forming its structural framework and maintaining its elasticity and strength. Additionally, elastin promotes cell interactions and signal transduction^[30], such as facilitating cell migration, growth, and proliferation^[31]. When UV radiation penetrates the epidermis and reaches the dermis, it induces the production of large amounts of reactive oxygen species, which stimulate the secretion of elastase. This leads to the degradation of elastin and various other components of the extracellular matrix, resulting in the destruction of skin structure, reduced mechanical strength, and the appearance of skin

laxity, wrinkles, and other photoaging-related symptoms^[32].

As shown in Figure 2c, unreacted chitosan exhibited significantly lower elastase inhibitory activity compared with the six CMRPs, and the elastase inhibition rate increased with increasing derivative concentration. When the concentration of the glucose increased from 4% to 6%, the elastase inhibition rate of the derivatives gradually increased from 62.70% to 90.91%. Similarly, when the concentration of the xylose increased from 1% to 2%, the elastase inhibition rate increased from 57.65% to 87.50%, showing a gradual upward trend. This trend was similar to that observed in Section 3.2.1 for DPPH and ABTS radical scavenging activity, suggesting that the accumulation of Maillard reaction products may contribute to inhibiting elastin degradation and maintaining skin structure. These results suggest that CMRPs are effective active substances for counteracting photoaging. However, the detailed mechanisms through which they exert their anti-photoaging effects require further investigation.

3.3 Effects of different CMRPs on UVB-induced HaCaT cells

3.3.1 Cell viability

The cytotoxicity of the six CMRPs on HaCaT cells was evaluated using the MTT assay. As shown in Figure 3a-f, within the dose range of 100–1600 µg/mL, all three chitosan-glucose Maillard derivatives (CG-4, CG-5, CG-6) exhibited cell viabilities greater than 80%, indicating no significant cytotoxic effects. Similarly, the three chitosan-xylose Maillard derivatives (CX-1, CX-1.5, CX-2) showed no significant cytotoxicity within the dose range of 200–800 µg/mL. Based on these results, the CMRPs were selected for subsequent experiments within the concentration range of 150–1600 µg/mL. The protective effect of different CMRPs was investigated using a UVB-irradiated photoaging HaCaT cell model. The results, shown in Figure 3g-l, revealed that after UVB irradiation, the cell viability in the model group was significantly lower than that in the control group ($P < 0.05$), indicating that the HaCaT cells had undergone photoaging damage. Intervention with the six CMRPs significantly increased the cell viability, suggesting that the end products provided a protection against photoaging damage in HaCaT cells. Notably, the maximum cell viability for the derivatives CG-4, CG-5, CG-6, CX-1, CX-1.5, and CX-2 was achieved within the dose range of 200–400 µg/mL, which was selected for subsequent experiments.

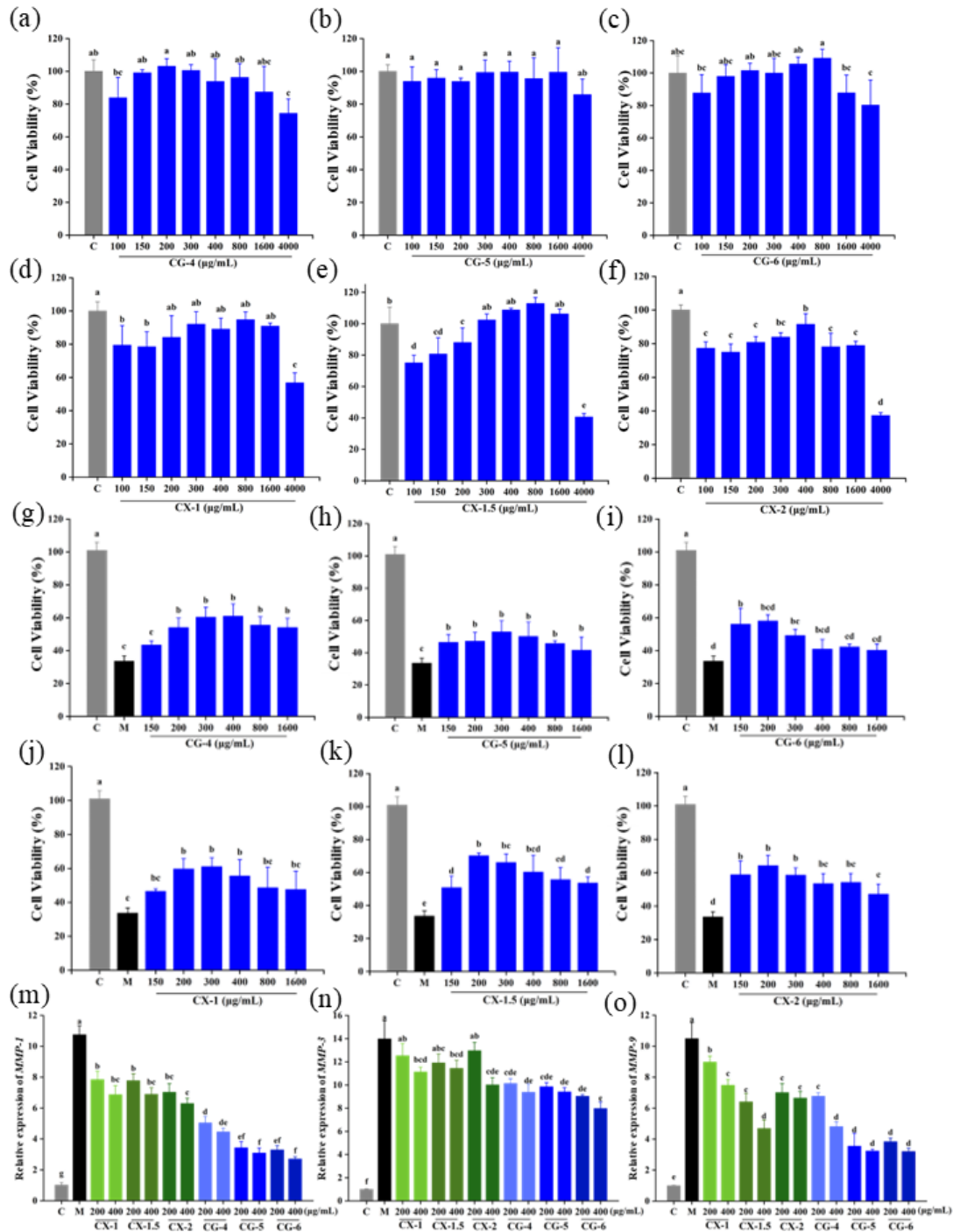


Figure 3 Toxic effect, cell viability, and MMPs gene expression of different CMRPs on HaCaT cells. (a-c) Toxic effect of chitosan-glucose Maillard derivatives; (d-f) Toxic effect of chitosan-xylose Maillard derivatives; (g-i) cell viability of chitosan-glucose Maillard derivatives; (j-l) cell viability of chitosan-xylose Maillard derivatives; (m) relative gene expression of *Mmp-1* on photoaging HaCaT cells; (n) relative gene expression of *Mmp-3* on photoaging HaCaT cells; (o) relative gene expression of *Mmp-9* on photoaging HaCaT cells.

^{a-g} Values with different letters are significantly different ($P < 0.05$).

3.3.2 Gene expression of matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases involved in tissue remodeling and extracellular matrix (ECM) degradation. Under UVB stimulation, skin cells secrete high amounts of MMPs, which degrade ECM collagen, resulting in reduced skin elasticity and firmness, ultimately leading to photoaging of the skin [33]. Based on their substrate specificities, the MMP family can be divided into five main subgroups, including collagenases, gelatinases, stromelysins, and others [34]. Among them, MMP-1 is an interstitial collagenase mainly secreted by epidermal and dermal cells, responsible for degrading type I and type III collagen in the skin. MMP-3 belongs to the stromelysin subgroup, primarily degrades elastin and type IV collagen at the epidermal-dermal junction and can also upregulate MMP-1 expression, further exacerbating skin laxity and reduced elasticity. MMP-9 is a gelatinase that degrades type IV collagen in the skin, disrupts skin structure, and is closely associated with photoaging [35]. Therefore, assessing the inhibitory effects of samples on the gene expression of MMP-1, MMP-3, and MMP-9 in cells can reflect their capacity to prevent collagen degradation and their anti-photoaging efficacy.

As shown in Figure 3m-o, after UVB treatment, the gene expression levels of *Mmp-1*, *Mmp-3*, and *Mmp-9* in the model group were significantly upregulated compared with the control group ($P < 0.05$), increasing to 10.75-fold, 13.98-fold, and 10.49-fold of the control group, respectively. After treatment with the six CMRPs, the gene expression of *Mmp-1*, *Mmp-3*, and *Mmp-9* was suppressed in a dose-dependent manner, indicating that all six derivatives could inhibit collagen degradation and exert anti-photoaging effects. At the concentration of 400 $\mu\text{g/mL}$, CG-6 showed the strongest inhibition of *Mmp-1*, *Mmp-3*, and *Mmp-9* gene expression, reducing their expression levels in cells to 25.16%, 57.11%, and 30.55% of the model group, respectively. At the same concentration, CX-1.5 showed the weakest inhibition of *Mmp-1* and *Mmp-3* gene expression, with expression levels reduced to 64.15% and 81.84% of the model group, respectively. CX-1 exhibited the weakest inhibition of *Mmp-9* gene expression, reducing its level to 71.36% of the model group. Overall, the chitosan-glucose Maillard derivatives (CG-4, CG-5, CG-6) exhibited stronger inhibitory effects on MMP expression compared to the chitosan-xylose Maillard derivatives (CX-1, CX-1.5, CX-2). According to section 3.1.1, it was found that the xylose derivative exhibited a faster reaction rate and a higher degree of Maillard reaction indicators, the superior anti-photoaging activity of CG-6 (the glucose CMRPs) may be attributed to the nature and composition of the Maillard reaction products formed. Generally, excessive progression of the Maillard reaction leads to the formation of high-molecular weight, brown polymers (melanoidins) with reduced antioxidant and biological activity compared with early- and intermediate-stage Maillard reaction products. Xylose, as an aldo-pentose, reacts more rapidly and tends to produce a greater proportion of advanced Maillard reaction products that may contain fewer active functional groups (e.g., free amino, carbonyl, or phenolic groups) responsible for antioxidative or anti-photoaging effects [18]. In contrast, glucose (an aldo-hexose) reacts more moderately, yielding Maillard reaction products enriched in intermediate products such as Amadori rearrangement compounds and low-molecular-weight heterocycles, which are known to exhibit stronger antioxidant, metal-chelating, and anti-photoaging properties [15, 36].

Taken together, CG-6 showed the best inhibitory effects on *Mmp-1*, *Mmp-3*, and *Mmp-9* expression, indicating that it is a promising functional factor with anti-photoaging potential. Therefore, the chitosan-glucose Maillard derivative CG-6 was selected for subsequent studies on the mechanism of its anti-photoaging effects.

3.3.3 ROS and inflammatory factor contents

When the skin is exposed to UVB radiation, large amounts of reactive oxygen species (ROS) are generated, inducing oxidative stress responses in the skin, which affects the expression of relevant protein kinases in the skin and directly or indirectly activates enzymes such as MMPs, hyaluronidase, and elastase, thereby degrading ECM components. Additionally, ROS activate downstream transcriptional pathways of various signaling pathways, disrupting skin cell functions and accelerating photoaging of the skin^[37, 38].

As shown in the figure 4a-b, under UVB stimulation, the fluorescence intensity of cells in the model group increased significantly, indicating that ROS levels were higher than those in the control group ($P < 0.05$), reaching 3.79 times the control level. This confirmed the occurrence of oxidative damage and photoaging. Following treatment with 200, 300, and 400 $\mu\text{g/mL}$ of the CMRPs CG-6, the intracellular ROS levels decreased in a dose-dependent manner, with all three concentrations showing significant differences compared to the model group ($P < 0.05$). Additionally, the intervention of 400 $\mu\text{g/mL}$ hyaluronic acid (HA) significantly reduced intracellular ROS levels compared to the model group ($P < 0.05$). Notably, treatment with 200 $\mu\text{g/mL}$ CG-6 exhibited a stronger ROS-scavenging effect than 400 $\mu\text{g/mL}$ HA. It was also found that Maillard reaction products extracted from coffee grounds significantly reduced intracellular ROS levels in Hela cells at both 2 h and 24 h post-treatment^[39]. These results suggest that the CG-6 can alleviate oxidative stress by reducing intracellular ROS levels, thereby exerting anti-photoaging effects.

The excessive production of ROS could stimulate the expression and secretion of pro-inflammatory factors such as interleukin-6 (IL-6)^[40], leading to immune dysregulation and inflammatory imbalance. Excessive production of IL-6 can disrupt the skin's immune barrier^[41], further promoting ECM collagen degradation and accelerating skin photoaging. Therefore, this section investigates the effect of CG-6 on IL-6 secretion in photoaged HaCaT cells.

As shown in Figure 4e, the level of IL-6 in HaCaT cells increased significantly ($P < 0.05$), reaching approximately 2.01 times that of the control group, indicating that UVB exposure induced inflammatory damage in skin cells. After treatment with 200, 300, and 400 $\mu\text{g/mL}$ CG-6, IL-6 levels significantly decreased compared with the model group ($P < 0.05$), with the lowest level reduced to 69.19% of the model group. These results indicate that CG-6 can repair inflammation-related damage in photoaged cells by reducing the intracellular levels of the pro-inflammatory factor IL-6.

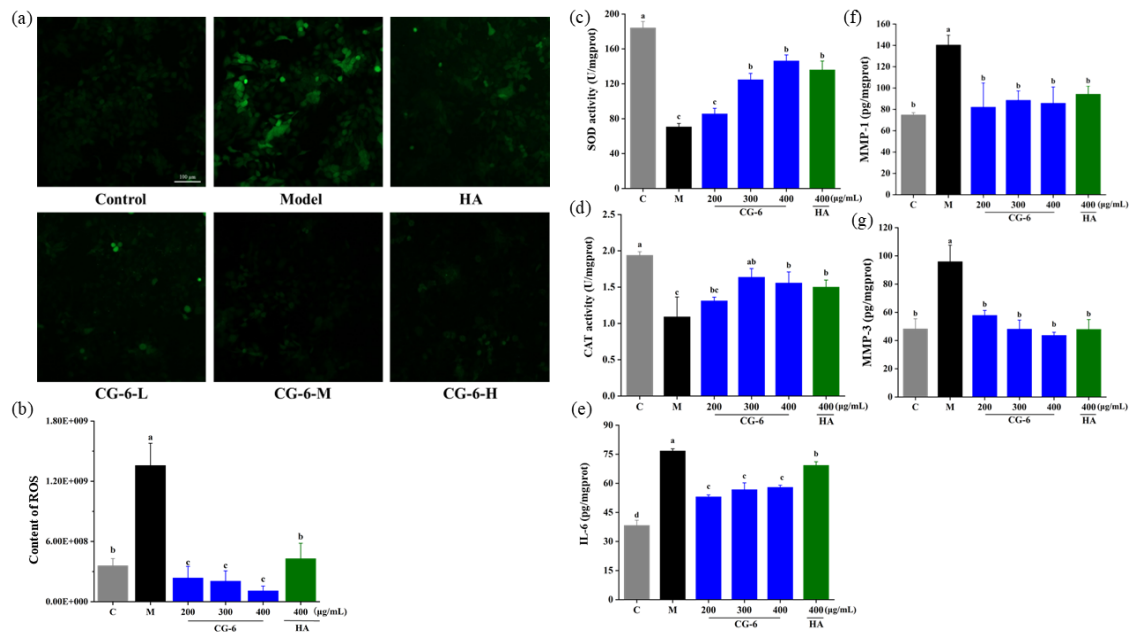


Figure 4 Effect of CG-6 on UVB-induced photoaging HaCaT cells. (a-b) ROS relative content; (c) SOD activity; (d) CAT activity; (e) IL-6 content; (f) MMP-1 content; (g) MMP-3 content

^{a-d} Values with different letters are significantly different ($P < 0.05$). HA is the abbreviations of hyaluronic acid (positive control drug).

3.3.4 MMPs content

As shown in Figure 4f, HaCaT cells exposed to UVB irradiation secreted significantly higher levels of MMP-1, with MMP-1 content in the model group increasing to 1.88 times that of the control group ($P < 0.05$), leading to collagen degradation in the skin. After treatment with various concentrations of CG-6 and HA, the MMP-1 content in cells was significantly reduced compared to the model group ($P < 0.05$), with no significant difference from the control group ($P > 0.05$). This indicates that the CG-6 can inhibit cellular secretion of MMP-1, thereby alleviating collagen degradation and exerting anti-photoaging effects. Figure 4g showed that UVB exposure also led to an increase of MMP-3, with MMP-3 levels in the model group rising to 1.99 times that of the control group ($P < 0.05$), resulting in reduced skin elasticity. Treatment with CG-6 and HA significantly decreased MMP-3 levels in photoaged cells compared to the model group ($P < 0.05$), with no significant difference compared to the control group ($P > 0.05$). Additionally, as the concentration of CG-6 increased, MMP-3 level was reduced to as low as 43.54% of the model group level. These results suggest that CG-6 effectively suppresses MMP-1 and MMP-3 secretion, thereby helping to maintain skin structural integrity and exerting anti-photoaging effects.

3.3.5 Antioxidant enzyme (antioxidase) activities

A protective network exists in human skin to resist damage caused by UV irradiation, consisting of endogenous antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). These endogenous antioxidants play an important role in eliminating excess ROS and maintaining the balance of the skin's "oxidation-antioxidation system." Under UVB stimulation, this balance is disrupted, the activity of antioxidant enzymes is impaired, and the skin's ability to resist oxidative stress decreases, leading to oxidative damage

and photoaging^[42].

The effects of the CMRPs CG-6 on SOD activity in photoaged HaCaT cells are shown in Figure 4c. Under UVB stimulation, SOD activity in the model group decreased to 38.32% of that in the control group ($P < 0.05$). Following intervention with CG-6 and HA, cellular SOD activity increased in a dose-dependent manner with rising CG-6 concentrations. At a concentration of 400 $\mu\text{g/mL}$, CG-6 increased SOD activity to 2.07 times that of the model group ($P < 0.05$), while treatment with the same concentration of HA also significantly enhanced SOD activity ($P < 0.05$), reaching 1.92 times that of the model group. These results suggest that CG-6 has a strong potential to repair oxidative damage. The effects of CG-6 on CAT activity in photoaged HaCaT cells are shown in Figure 4d. In the model group, CAT activity decreased to 56.26% of that in the control group ($P < 0.05$), indicating the occurrence of oxidative stress. After treatment with 200, 300, and 400 $\mu\text{g/mL}$ CG-6 led to a significant increase in CAT activity compared to the model group, with 300 $\mu\text{g/mL}$, restoring CAT activity to level comparable to the control group, reaching 1.50 times that of the model group. In summary, CG-6 enhances antioxidant enzymes activity in photoaged HaCaT cells, improving cellular resistance to oxidative stress and exerting anti-photoaging effects.

3.4 Effects of different CMRPs on the expression of related genes in MAPK signaling pathway (in HaCaT cells)

The mitogen-activated protein kinase (MAPK) pathway is a signaling cascade closely associated with oxidative stress and pro-inflammatory responses^[43]. UV radiation can activate the MAPK cascade, leading to skin photoaging and related diseases^[44]. The MAPK signaling pathway includes three major kinases: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK^[45]. When the skin is exposed to UV stimulation, the MAPK pathway is activated, resulting in phosphorylation of ERK, JNK, and p38. These phosphorylation kinases then transmit signals to c-Jun, thereby increasing its protein levels. c-Jun forms heterodimers with c-Fos or homodimers with itself, constituting the transcription factor activator protein-1 (AP-1)^[46]. Upregulation of AP-1 accelerates collagen degradation in the extracellular matrix (ECM), as activated AP-1 translocates to the nucleus and further increases MMPs expression. Additionally, AP-1 can block transforming growth factor (TGF)-mediated collagen synthesis in the ECM, thereby accelerating skin photoaging. Interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) are released in large amounts upon UV stimulation, activating downstream pro-inflammatory pathways such as p38 MAPK and the JNK pathway, which leads to skin inflammation and damage, and these pro-inflammatory factors can directly induce keratinocyte death^[47]. NF- κ B and p53 are downstream transcription factors of the MAPK pathway; upon UV activation, NF- κ B translocates to the nucleus in skin cells^[48], where it promotes MMPs expression and stimulates the secretion of pro-inflammatory factors through transcriptional mechanisms, accelerating ECM collagen degradation.

The effect of CG-6 on the gene expression of *Mmp-1*, *Mmp-3*, and *Mmp-9* in photoaged cells is shown in Figure 5a-c. After UVB irradiation, the expression levels of *Mmp-1*, *Mmp-3*, and *Mmp-9* in the model group were significantly upregulated to 6.45-fold, 10.65-fold, and 9.63-fold of the control group, respectively,

indicating successful model establishment ($P < 0.05$). After treatment with CG-6 and HA at the same concentration of 400 $\mu\text{g/mL}$, *Mmp-1* expression was reduced to 53.73% and 70.26% of the model group, respectively. *Mmp-3* expression was reduced to 46.03% and 58.86%, and *Mmp-9* expression was reduced to 11.48% and 49.62%, respectively. In summary, CG-6 dose-dependently downregulated the expression of *Mmp-1*, *Mmp-3*, and *Mmp-9* genes in cells, demonstrating superior or comparable effects to HA at the same concentration. These results are consistent with the protein expression findings discussed in section 3.3.2.

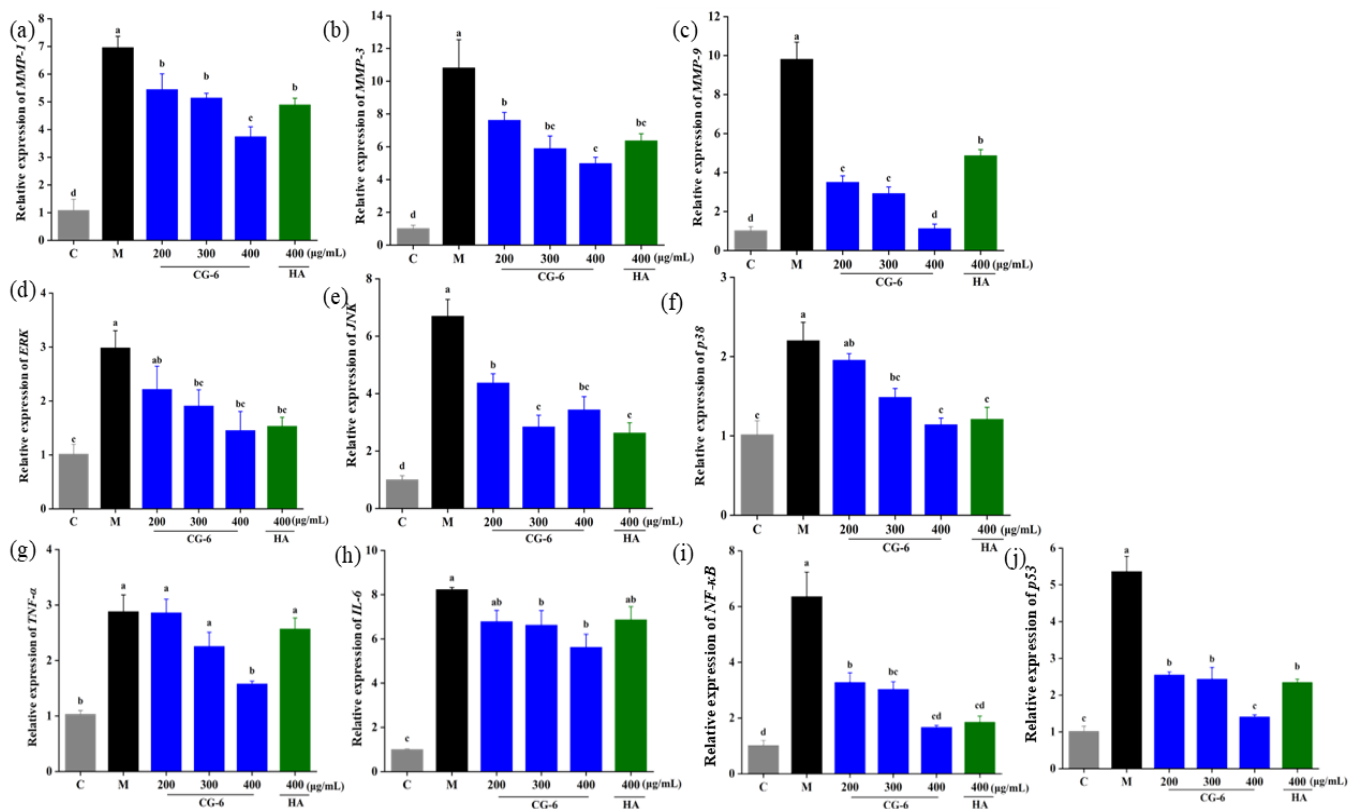


Figure 5 Effect of CG-6 on related gene expression levels of MAPK signaling pathway in HaCaT cells. (a) *Mmp-1*; (b) *Mmp-3*; (c) *Mmp-9*; (d) *Erk*; (e) *Jnk*; (f) *p38*; (g) *Tnf-α*; (h) *Il-6*; (i) *Nf-κB*; (j) *p53*.

^{a-d} Values with different letters are significantly different ($P < 0.05$).

As shown in Figure 5d-f, after UVB irradiation, the expression levels of *Erk*, *Jnk*, and *p38* genes were significantly upregulated compared with the control group ($P < 0.05$), reaching 2.94-fold, 6.70-fold, and 2.17-fold of the control group, respectively. The intervention with different concentrations of CG-6 at 200, 300, and 400 $\mu\text{g/mL}$ or 400 $\mu\text{g/mL}$ of HA, the expression levels of these genes were significantly reduced compared to the model group ($P < 0.05$). Specifically, CG-6 treatment reduced *Erk* expression to 48.71% of the model group, while HA reduced it to 51.37%. CG-6 reduced *Jnk* expression to a minimum of 42.39% of the model group, while HA reduced it to 39.25%. *p38* expression was also downregulated, with CG-6 and HA treatments at 400 $\mu\text{g/mL}$ reducing *p38* expression to 51.85% and 54.99% of the model group, respectively. CG-6 significantly downregulated the expression of *Erk*, *Jnk*, and *p38* in HaCaT cells, with a similar effect to those of HA at the same concentration.

As shown in Figure 5g-h, after UVB irradiation, the gene expression of the inflammatory factor *Tnf-α*

and *Il-6* was significantly upregulated ($P < 0.05$), reaching 2.80-fold and 8.23-fold of the control group, respectively. Intervention with 200, 300, and 400 $\mu\text{g/mL}$ CG-6 or 400 $\mu\text{g/mL}$ HA, the expression levels of both genes showed a downregulation trend. After CG-6 treatment, the expression of *Tnf- α* decreased in a dose-dependent manner, with the lowest expression reduced to 54.75% of the model group. HA intervention reduced *Tnf- α* expression to 89.16% of the model group. Different concentrations of CG-6 reduced the expression of *Il-6* to 82.41%, 80.49%, and 68.32% of the model group, respectively, while treatment with 400 $\mu\text{g/mL}$ HA reduced it to 83.43% of the model group. These results are consistent with the trends observed in section 3.3.3, indicating that CG-6 can exert anti-photoaging effects by downregulating the expression of inflammation-related genes induced by UVB.

As shown in Figure 5i-j, after UVB irradiation, the gene expression of *Nf- κ b* and *p53* was significantly upregulated ($P < 0.05$), reaching 6.26-fold and 5.29-fold of the control group, respectively. After treatment with 400 $\mu\text{g/mL}$ CG-6 and HA, the expression levels of *Nf- κ b* were reduced to 26.18% and 29.07% of the model group, respectively. Taken together, CG-6 can dose-dependently downregulate the expression of downstream *Nf- κ b* and *p53* in the MAPK pathway, thereby inhibiting MMPs expression to exert anti-photoaging effects. These results suggest that CG-6 may exert anti-photoaging effects by regulating the expression of key targets in the MAPK signaling pathway.

3.5 Toxic evaluation of CMRPs CG-6 in vivo

3.5.1 Effects of CG-6 on body weight and organ index

The effects of CG-6 on the body weight of male and female mice are shown in Figure 6a. During the experiment period, the body weight of mice in all groups gradually increased. On the last day of the experiment, the body weight of mice in the CB group was significantly higher than that on the first day ($P < 0.05$), reaching 137.80% of the initial weight. Similarly, the LB, MB, and HB groups showed significant increases in body weight compared to the first day ($P < 0.05$), with no significant differences compared with the CB group ($P > 0.05$). Mice in the CG, LG, MG, and HG groups also exhibited significant increases in body weight compared to their initial weight ($P < 0.05$), and no significant differences were observed among these groups ($P > 0.05$). These results suggest that oral administration of CG-6 at doses of 1.0, 2.0, and 3.0 g/kg bw/day for four weeks does not induce abnormal weight gain or loss in male or female mice and does not negatively effects on normal growth and development.

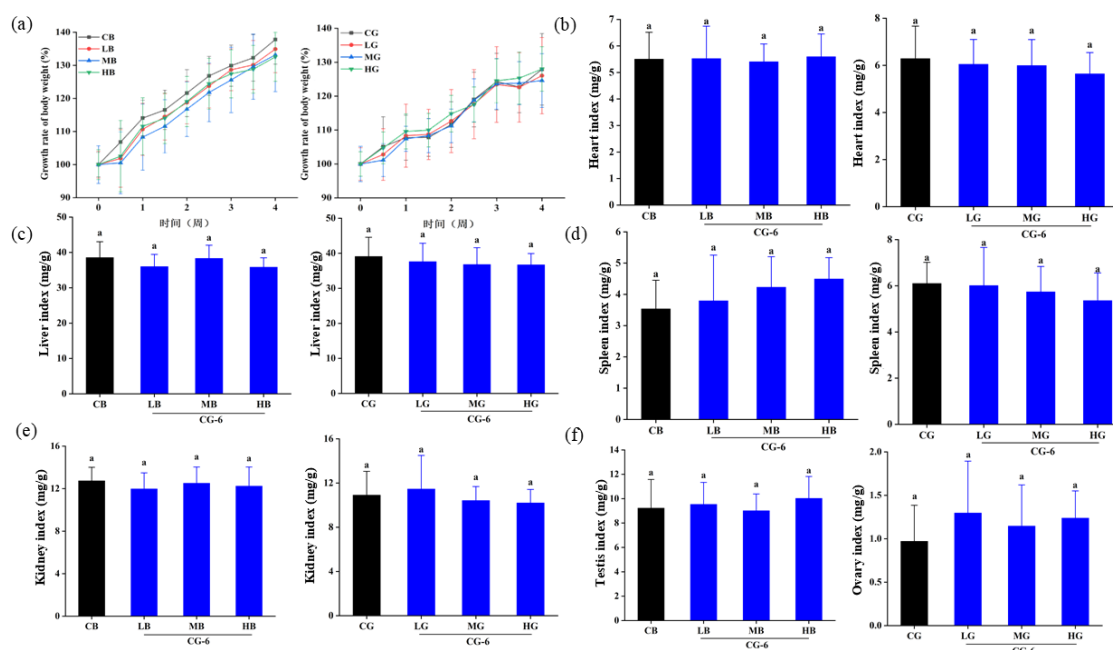


Figure 6 Body weight and organ index of mice, (left) male indicators; (right) female indicators. (a) body weight of mice; (b) heart index; (c) liver index; (d) spleen index; (e) kidney index; (f) testis (left) and ovary (right) index.

The organ indices of the heart, liver, spleen, kidneys, and reproductive organs (testes/ovaries) of male and female mice are shown in Figure 6b-f. Exposure to potentially toxic substances may result in toxic injury in mice, leading to pathological changes such as cardiac hypertrophy, hyperplasia, or atrophy. As shown in Figure 6b, there were no significant differences ($P > 0.05$) in the heart organ indices between the sample groups and their respective gender-matched control groups. Furthermore, no abnormalities such as shrinkage or enlargement were observed in the gross morphology of the heart upon dissection. The liver, spleen, and kidneys are major metabolic organs involved in maintaining homeostasis and are sensitive indicators of toxic effects. According to Figure 6c-e, there were no significant differences ($P > 0.05$) in the organ indices of the liver, spleen, and kidneys between the sample groups and their respective control groups. In addition, gross examination of these organs revealed no visible abnormalities such as shrinkage, edema, or hypertrophy. The testes and ovaries are the reproductive organs of male and female mice, respectively, and reflect the health of the endocrine and reproductive systems. As shown in Figure 6f, no significant differences ($P > 0.05$) were observed in the organ indices of the testes and ovaries between the sample groups and their gender-matched controls. Gross anatomical inspection of the testes and ovaries showed no signs of texture alteration, swelling, or mass formation. These results indicate that CG-6 did not induce any observable pathological changes in the major organs of the mice.

3.5.2 Effects of the CG-6 on organ tissue morphology

Histological morphology of the liver, kidneys, and spleen was examined using hematoxylin and eosin (H&E) staining to determine whether pathological changes were present in these organs. As shown in Figure 7a, the livers of mice in all groups exhibited clearly defined margins, firm texture, reddish-brown color, smooth and glossy surfaces, with no visible depressions, masses, or rupture marks. In the CB and CG groups, histological sections of liver tissue showed normal appearance of the central vein and portal area, with

hepatocytes arranged in an orderly fashion. Similarly, in the LB, MB, HB, LG, MG, and HG groups, the central and portal vein walls remained intact. Hepatocytes were radially arranged around the central vein with clear cell boundaries, normal spacing, and no abnormalities in nuclear size or positioning. The nuclei were distinct and showed no signs of shrinkage or dissolution. No significant steatosis, fibrotic accumulation, or inflammatory cell infiltration was observed in the liver cells of male or female mice in any group. Lobular architecture remained intact in the control and all dose groups.

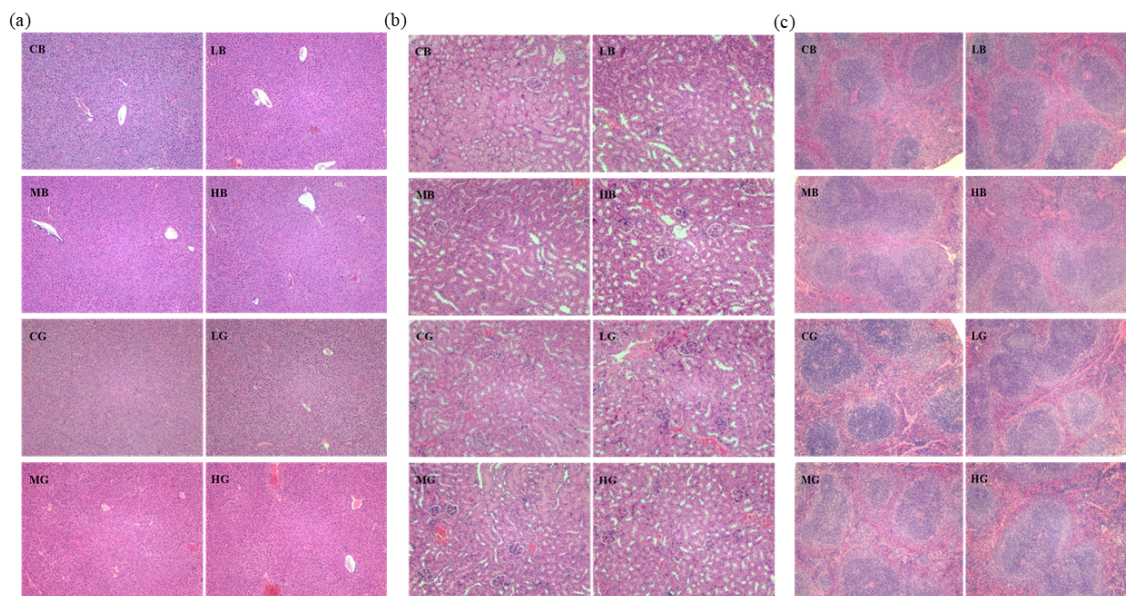


Figure 7 Organ tissue morphology of CG-6-treated mice. (a) liver tissue; (b) kidney tissue; (c) spleen tissue

As shown in Figure 7b, the kidneys of all mice were bean-shaped, bright red in color, and covered with a thin capsule. Both kidneys were uniform in size and shape, with smooth surfaces free of ulcers, masses, or hemorrhagic spots. In the CB and CG groups, histological sections showed normal glomerular and tubular structures. Following CG-6 administration, glomeruli remained structurally intact without evidence of shrinkage or deformation. Renal tubular epithelial cells were clearly defined, with no signs of swelling, vacuolar degeneration, or cell disintegration. No inflammatory cell infiltration or signs of interstitial edema were observed in the renal interstitium of any group.

As shown in Figure 7c, the spleens of all mice were elongated, dark red, and covered by a capsule. No signs of rupture, shrinkage, or discoloration were observed in any group. In the CB and CG groups, the splenic capsule and trabeculae remained intact. Histological sections of the spleen in the LB, MB, HB, LG, MG, and HG groups indicated that after CG-6 administration, splenic structures were preserved without evidence of trabecular degeneration. In all groups, including the control and the low-, medium-, and high-dose groups, the white and red pulp were structurally intact and clearly delineated, with no marked reduction in lymphocyte numbers. No significant inflammatory infiltration, fibrosis, or necrosis was observed.

In summary, CG-6 did not induce significant pathological alterations in the liver, kidneys, or spleen of male or female mice, which is consistent with the findings in Section 3.5.1 and the gross anatomical observations.

3.5.3 Effects of the CG-6 on complete blood count (CBC) index

Hematological parameter testing is an important approach to assessing the health status of experimental animals. The effects of CG-6 on the hematological indices of male and female mice are shown in Table 2 and Table 3. After 28 days of oral administration of CG-6 at low, medium, and high doses, there were no significant changes ($P > 0.05$) in red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), or mean corpuscular hemoglobin concentration (MCHC) in the LB, MB, and HB groups compared to the CB group. Similarly, no significant increases or decreases in these parameters were observed in the LG, MG, and HG groups compared to the CG group ($P > 0.05$). These findings indicate that CG-6 does not induce abnormalities in hematopoietic function, blood viscosity, or red blood cell morphology in mice. Furthermore, leukocyte (LEU) and lymphocyte (LYM) counts in the low-, medium-, and high-dose groups showed no significant differences from those of their respective control groups (CB and CG), suggesting that CG-6 does not induce an inflammatory stress response and that the endocrine and immune systems of the mice remained stable and balanced. Indicators reflecting coagulation function and platelet volume, including platelet count (PLT), platelet distribution width (PDW), and mean platelet volume (MPV), also showed no significant differences ($P > 0.05$) between CG-6-treated groups and the untreated controls after 28 days of administration.

Table 2 Effect of CG-6 on blood routine in male mice

	CB	LB	MB	HB
RBC ($\times 10^{12}/L$)	10.22 $\pm$ 0.55 ^a	9.96 $\pm$ 0.49 ^a	10.09 $\pm$ 0.61 ^a	9.934 $\pm$ 0.78 ^a
HGB (g/L)	153.17 $\pm$ 5.30 ^a	145.50 $\pm$ 8.74 ^a	149.31 $\pm$ 10.07 ^a	148.83 $\pm$ 10.15 ^a
HCT (%)	49.01 $\pm$ 1.75 ^a	46.08 $\pm$ 2.88 ^a	47.45 $\pm$ 3.63 ^a	46.50 $\pm$ 3.66 ^a
MCV (fL)	48.04 $\pm$ 2.12 ^a	46.23 $\pm$ 1.34 ^a	47.01 $\pm$ 1.78 ^a	46.82 $\pm$ 1.61 ^a
MCH (pg)	15.02 $\pm$ 0.77 ^a	14.59 $\pm$ 0.34 ^a	14.80 $\pm$ 0.56 ^a	14.99 $\pm$ 0.40 ^a
MCHC (g/L)	312.69 $\pm$ 9.49 ^a	315.93 $\pm$ 8.52 ^a	314.96 $\pm$ 8.30 ^a	320.49 $\pm$ 10.45 ^a
WBC ($\times 10^9/L$)	3.91 $\pm$ 0.65 ^a	4.02 $\pm$ 1.24 ^a	4.05 $\pm$ 0.62 ^a	4.09 $\pm$ 1.15 ^a
LYM (%)	69.44 $\pm$ 7.29 ^a	73.01 $\pm$ 3.00 ^a	70.24 $\pm$ 6.02 ^a	70.98 $\pm$ 6.52 ^a
PLT ($\times 10^9/L$)	983.92 $\pm$ 224.86 ^a	992.10 $\pm$ 195.51 ^a	975.75 $\pm$ 134.92 ^a	978.5 $\pm$ 144.97 ^a
PDW (%)	11.5 $\pm$ 1.22 ^a	11.98 $\pm$ 0.61 ^a	11.46 $\pm$ 1.71 ^a	11.65 $\pm$ 1.00 ^a
MPV (fL)	8.97 $\pm$ 0.54 ^a	9.09 $\pm$ 0.42 ^a	8.81 $\pm$ 0.50 ^a	9.13 $\pm$ 0.41 ^a

Table 3 Effect of CG-6 on blood routine in female mice

	CG	LG	MG	HG
RBC ($\times 10^{12}/L$)	10.37 $\pm$ 0.53 ^a	10.35 $\pm$ 0.54 ^a	10.15 $\pm$ 0.39 ^a	10.16 $\pm$ 0.43 ^a
HGB (g/L)	157.30 $\pm$ 7.77 ^a	157.09 $\pm$ 7.43 ^a	154.58 $\pm$ 5.59 ^a	155.10 $\pm$ 6.66 ^a
HCT (%)	51.24 $\pm$ 2.74 ^a	49.96 $\pm$ 2.90 ^a	49.75 $\pm$ 1.98 ^a	49.19 $\pm$ 2.01 ^a
MCV (fL)	49.41 $\pm$ 1.37 ^a	48.31 $\pm$ 1.68 ^a	49.04 $\pm$ 1.59 ^a	48.42 $\pm$ 0.99 ^a
MCH (pg)	15.18 $\pm$ 0.67 ^a	15.19 $\pm$ 0.47 ^a	15.24 $\pm$ 0.37 ^a	15.27 $\pm$ 0.44 ^a
MCHC (g/L)	307.37 $\pm$ 14.35 ^a	314.75 $\pm$ 9.75 ^a	310.91 $\pm$ 9.71 ^a	315.38 $\pm$ 8.07 ^a
WBC ($\times 10^9/L$)	7.10 $\pm$ 1.58 ^a	7.12 $\pm$ 1.63 ^a	7.57 $\pm$ 1.23 ^a	7.60 $\pm$ 1.73 ^a
LYM (%)	81.84 $\pm$ 3.95 ^a	80.07 $\pm$ 3.33 ^a	81.25 $\pm$ 5.11 ^a	81.26 $\pm$ 2.50 ^a
PLT ($\times 10^9/L$)	1010.45 $\pm$	937.92 $\pm$	1009.54 $\pm$	984.43 $\pm$
PDW (%)	10.84 $\pm$ 1.19 ^a	10.50 $\pm$ 1.14 ^a	10.75 $\pm$ 1.40 ^a	10.73 $\pm$ 0.88 ^a
MPV (fL)	8.67 $\pm$ 0.61 ^a	8.44 $\pm$ 0.46 ^a	8.63 $\pm$ 0.65 ^a	8.69 $\pm$ 0.34 ^a

In conclusion, the intake of CMRPs CG-6 did not impair hematopoietic or coagulation functions in mice. No disturbances were observed in immune status or endocrine system balance across any dose group, and no signs of hematological disorders were detected.

3.5.4 Effects of the CG-6 on serum biochemistry index

The effects of CG-6 on liver function in mice were evaluated by quantitatively analyzing serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin (ALB), and total protein (TP)^[49]. Renal function was assessed by measuring serum urea nitrogen (UREA) and creatinine (CREA) levels^[50]. Additionally, changes in serum glucose (GLU), triglycerides (TG), and total cholesterol (TC) were analyzed to determine the impact of CG-6 on glucose and lipid metabolism in mice^[51].

As shown in Figure 8a, after the oral administration of CG-6, the serum ALT activity in the low-, medium-, and high-dose groups exhibited slight fluctuations compared with the corresponding sex-matched control groups, but no significant differences were observed ($P > 0.05$). Similarly, as shown in Figure 8b, the serum AST activity in each CG-6 dose group showed no significant difference from the respective control groups ($P > 0.05$). To further assess the effect of CG-6 on liver function, the AST/ALT ratio in mouse serum was calculated, as shown in Figure 8c. The results showed that the AST/ALT ratio in the LB, MB, and HB groups did not increase compared to the CB group, and no significant differences were observed ($P > 0.05$). Similarly, no increase in the AST/ALT ratio was observed in female mice following CG-6 treatment ($P > 0.05$). These findings suggest that CG-6 does not disrupt liver cell structure or induce liver injury in mice. In the liver, alkaline phosphatase (ALP) is involved in bile salt production and promotes lipid digestion and absorption. As shown in Figure 8d, ALP activity in the serum of male and female mice showed no significant increase or decrease after CG-6 administration at various doses compared to the corresponding CB and CG groups ($P > 0.05$), indicating that CG-6 intake did not lead to anorexia or malnutrition. A similar trend was observed for total protein (TP) levels: oral administration of CG-6 did not cause significant changes in TP levels ($P > 0.05$). Figure 8f showed the serum albumin (ALB) levels in each group. No significant differences were found in ALB levels between the LG, MG, HG groups and the CG group in female mice ($P > 0.05$). However, the ALB level in the HB group was significantly higher than in the CB group ($P < 0.05$). ALB is a major antioxidant in the blood and is associated with enhanced immune capacity.

Taken together with the histopathological liver findings in Section 3.5.2 and the serum levels of ALT, AST, ALP, TP, and ALB, these results indicate that oral administration of CG-6 at low, medium, and high doses does not significantly affect liver function in male or female mice, does not increase hepatic burden, and does not induce liver injury such as hepatitis or hepatic fibrosis.

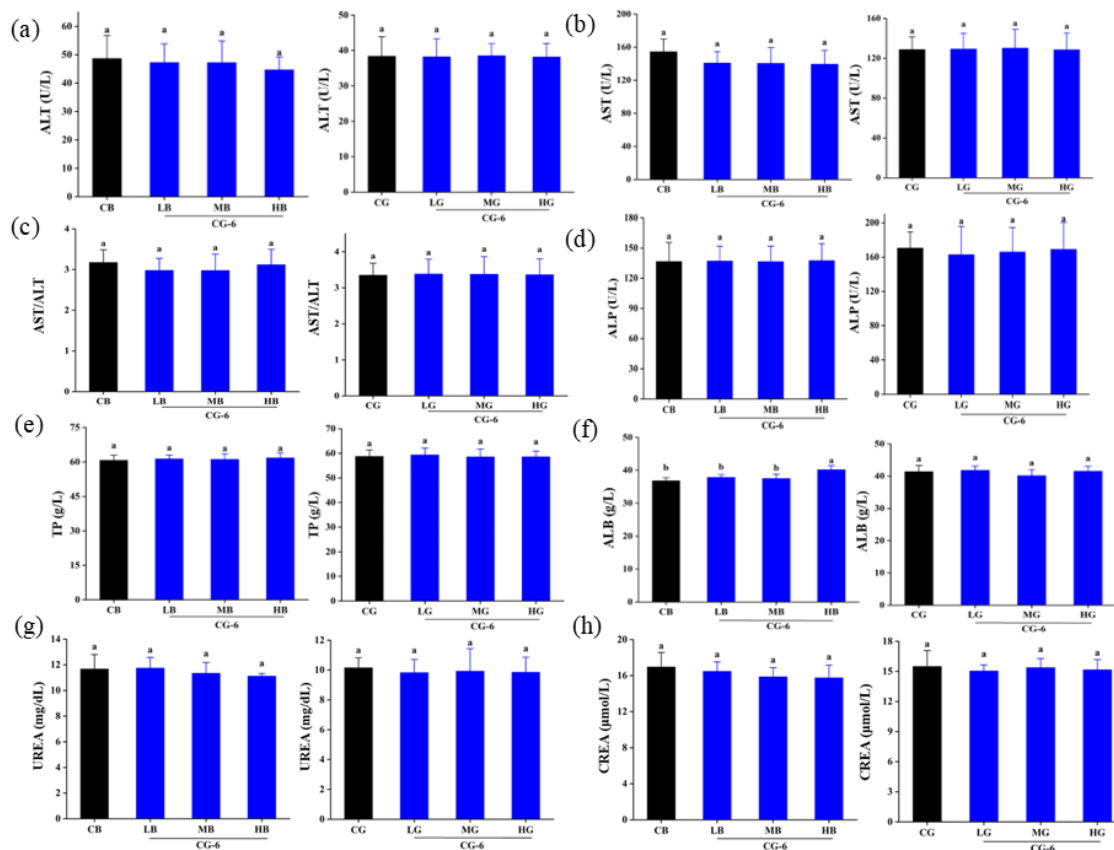


Figure 8 Effects of CG-6 on liver and kidney function-related indexes in mice, (left) male indicators; (right) female indicators. (a) ALT; (b) AST; (c) AST/ALT; (d) ALP; (e) TP; (f) ALB; (g) UREA; (h) CREA

As shown in Figure 8g-h, following intervention with different doses of CG-6, the serum UREA levels in both male and female mice exhibited slight fluctuations compared to the corresponding CB and CG control groups, but no significant differences were observed ($P > 0.05$), indicating that CG-6 intake does not impair glomerular filtration function. A similar trend was observed for serum CREA levels; oral administration of CG-6 did not result in significant increases or decreases in CREA concentration ($P > 0.05$). Taken together with the renal histopathological findings in Section 3.5.2, these results suggest that administration of low, medium, and high doses of CG-6 has no significant impact on renal function indicators in male and female mice and does not impair the kidney's filtration and metabolic functions.

As shown in Figure 9a, after intervention with various doses of CG-6, the serum glucose (GLU) levels in both male and female mice showed slight fluctuations compared to the corresponding CB and CG groups, but no significant differences were observed ($P > 0.05$), indicating that CG-6 intake does not disrupt the body's glucose metabolism balance. A similar trend was observed in the serum triglyceride (TG) and total cholesterol (TC) levels: oral administration of CG-6 did not result in significant increases or decreases in TG and TC concentrations ($P > 0.05$). The results in section 3.5.1 also demonstrate that CG-6 does not lead to abnormal weight gain or loss in mice, suggesting that oral administration of CG-6 at low, medium, and high doses does not impair glucose and lipid metabolic homeostasis in either male or female mice.

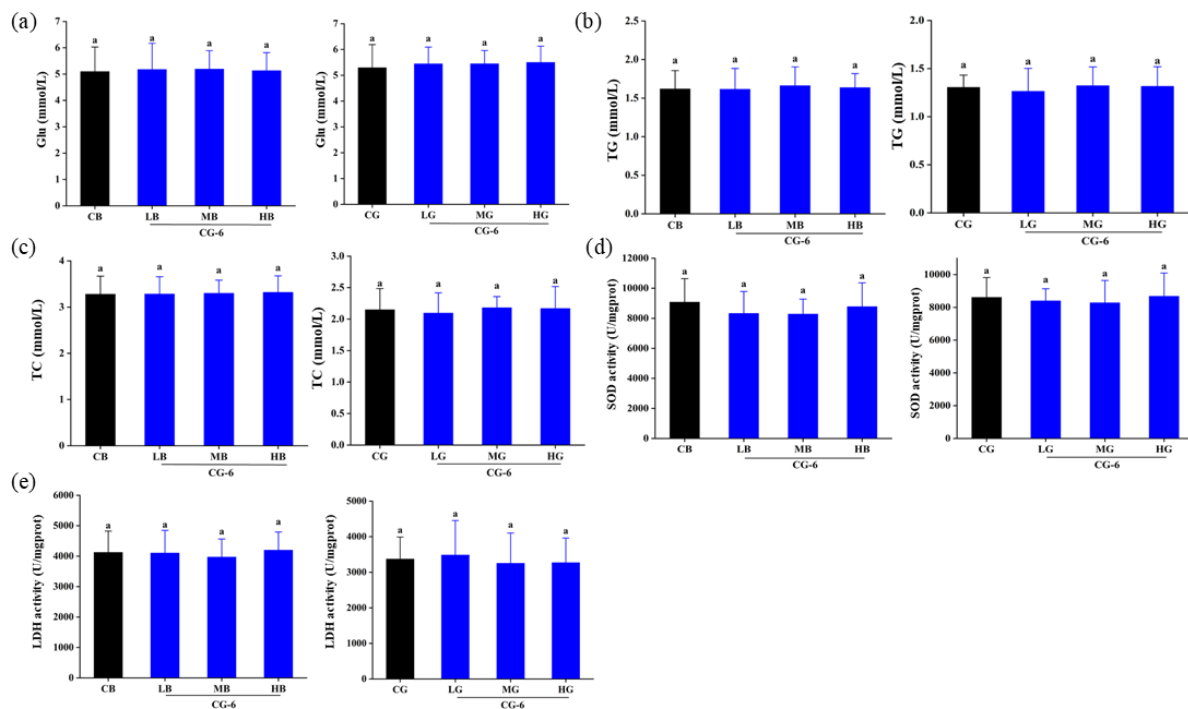


Figure 9 Effects of CG-6 on blood glucose, lipid metabolism and the activity of liver antioxidantase in mice, (left) male indicators; (right) female indicators. (a) blood glucose; (b) TG concentration; (c) TC concentration; (d) SOD activity; (e) LDH activity.

3.5.5 Effects of the CG-6 on liver antioxidantase

Superoxide dismutase (SOD) is a key antioxidant enzyme in the liver that protects against damage caused by viruses and drugs by scavenging free radicals. The activity level of SOD directly affects the liver's ability to resist external stimuli, a reduction in SOD activity during liver injury can hinder liver function repair, while the accumulation of excessive free radicals may further exacerbate liver dysfunction [52]. Lactate dehydrogenase (LDH), which is widely distributed throughout the body and highly active in the heart, liver, and kidneys, participates in carbohydrate metabolism by catalyzing the conversion of lactate to pyruvate. When diseases such as viral hepatitis, drug-induced liver injury, or alcoholic hepatitis occur, the integrity of hepatocytes is compromised, leading to the release of intracellular LDH [53].

The hepatic SOD activity in the CB and CG groups was 9075 and 8610 U/mg protein, respectively. As shown in Figure 9d, after 28 days of oral administration of CG-6, the SOD activity in mouse liver tissues exhibited slight fluctuations, but there were no significant differences in SOD activity between male and female mice in the low-, medium-, and high-dose groups compared to their respective sex-matched control groups ($P > 0.05$), indicating that CG-6 does not impair the liver's ability to resist external stimuli. The effect of CG-6 on LDH activity in mouse liver tissue is shown in Figure 9e. In the CB and CG groups, hepatic LDH activity was 4121 and 3369 U/g protein, respectively. After 28 days of CG-6 gavage, the LDH activity in liver tissues showed some fluctuation, however, the average LDH activity in the livers of male mice in the low-, medium-, and high-dose groups showed no significant difference compared with the CB group ($P > 0.05$). The same trend was observed in female mice, with no significant differences in LDH activity between the CG group and any of the CG-6 dose groups ($P > 0.05$). In summary, continuous gavage of CG-6 for 28 days did

not reduce the hepatic SOD or LDH enzyme activities in male or female mice. These findings are consistent with the liver histopathological results in section 3.5.2 and the serum liver function indicators in section 3.5.4, indicating that CG-6 does not cause liver damage.

4. Conclusions

In this study, chitosan-glucose and chitosan-xylose Maillard reaction products were prepared through a moist-heat method and the products were characterized. The CMRPs with the strongest anti-photoaging activity was selected, and its underlying mechanism was investigated. A 28-day oral toxicity experiment in mice was conducted to assess the *in vivo* safety of the chitosan Maillard derivative CG-6. Results showed that xylose accumulated Maillard reaction products more rapidly than glucose. As the monosaccharide concentration increased, the CMRPs exhibited enhanced DPPH and ABTS radical scavenging abilities as well as stronger elastase inhibition activity. In a UVB-induced photoaging model using HaCaT cells, CG-6 demonstrated high anti-photoaging activity, significantly suppressing the gene expression of MMPs. Further mechanistic studies revealed that CG-6 effectively reduced ROS accumulation in photoaged HaCaT cells, enhanced the activities of antioxidant enzymes SOD and CAT, and inhibited the expression of *Il-6*, *Mmp-1*, and *Mmp-3* genes, thereby counteracting oxidative stress and preventing collagen degradation. Additionally, CG-6 regulated the expression of key genes in the MAPK signaling pathway to exert its anti-photoaging effects. In the chronic toxicity evaluation, CG-6 showed no adverse effects on mouse health, indicating good safety and potential for use in oral anti-photoaging formulations.

Considering its excellent *in vitro* efficacy and oral safety, CG-6 is presumed to act as a multifunctional anti-photoaging agent *in vivo* by enhancing endogenous antioxidant defense systems and suppressing pro-inflammatory cytokines. Moreover, the bioactive Maillard-derived oligosaccharides and amino-carbonyl compounds in CG-6 could potentially influence the gut–skin axis by improving intestinal barrier integrity and reducing oxidative metabolites that contribute to skin aging. In the follow-up study, we will investigate the protective effects and mechanisms of CG-6 against skin photoaging *in vivo*.

Declaration of competing interest

The authors declare that they have not known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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