

Repairing and Protecting Effects of *Pneumatophorus japonicus* heads peptides on GES-1 Cells Damaged by Alcohol

DOI: 10.25177/JFST.8.1.RA.10836

Research

Accepted Date: 4th May 2023; Published Date: 10th May 2023

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CITATION

Yi-Ying Tang, Ming-Hui Gao, Jie-Shuai Zhang, Ning-ping Tao, Xi-Chang Wang, Ping Wang, Shang-Gui Deng, Miao-Si Hong, Ying Lu, Jing-Ya Yang, Chang-Hua Xu, Repairing and Protecting Effects of *Pneumatophorus japonicus* heads peptides on GES-1 Cells Damaged by Alcohol(2023) Journal of Food Science & Technology 8(1) : 479-491

ABSTRACT

Stomach mucosal damage posed a great impact on stomach health and may escalated to stomach cancer. Given that gastric mucosal epithelial cells (GES-1) were the foremost essential part of gastric mucosal defense barrier, their repair and protection were crucial for gastric strengthening. In this study, based on "macro-micro-nano-level" molecular spectroscopy, a multi-scale in-situ analysis approach of microscopic imaging, complemented by cellular biological methods, was proposed to systematically investigate the dynamic regulation of home-developed *Pneumatophorus japonicus* head peptides (PHPs) on the visual-chemical structure and composition distribution of alcohol-damaged GES-1, and subsequently to reveal the interaction mechanism between PHPs and GES-1 components. The results showed that 3-10 kDa PHPs had superior repair and protection effects compared to gastric mucin, significantly promoted cell proliferation, reduced the leakage of lactate dehydrogenase (LDH), and increased the mitochondrial membrane potential (MMP). It was deduced that PHPs promoted synthesis of glycoproteins, enhanced superoxide dismutase (SOD) and inhibited production of reactive oxygen species (ROS), thus achieving protection and repair functions on GES-1. This study provided theoretical and technical reference for the development of new natural gastric repair peptide products with high activity in future.

Keywords: *Pneumatophorus japonicus*; GES-1; FTIR; Infrared imaging; Alcohol; Repairing effect

1. INTRODUCTION

Acute gastric injury and chronic gastric ulcer were currently two major categories of gastric injury, the most common of which was acute gastric injury caused by irritating drugs or food, bacterial infection, etc. that damaged gastric mucosal barrier, mainly manifested as gastric mucosal bleeding or congestion, erosion edema and other symptoms. At present, clinically common types of gastric diseases were predominantly caused by lesions of the gastric mucosa, and alcohol-induced gastric mucosal damage was a refractory disease that frequently occurred clinically, including gastric ulcers, gastritis, stress gastric mucosal lesions, etc. [1]. Gastric mucosa played an essential role in maintaining gastric physiological functions. As a gastric barrier, gastric mucosa shields deep tissues from damage by gastric fluid components as well as exogenous gastric mucosal irritants [2]. Damage to gastric mucosa posed a serious risk to gastric health, which worsened to gastric cancer, therefore, protection of gastric mucosa was of utmost importance for stomach health.

In clinical practice, pharmacological treatments used to repair the gastric mucosa were primarily gastric mucosal protective agents, which were classified into gastrointestinal hormones, sulfur hydrogen bonds, bismuth agents, columnar cell stabilizers, and others according to the drug structure and mechanism of action. It was demonstrated that bismuth agents facilitated bicarbonate secretion, improved blood circulation of gastric mucosa, and enhanced its defense function [3]; unfortunately, darkening of stools and transient discoloration of teeth might occur during drug administration [4]. Sucralfate enhanced mucus secretion and stimulated synthesis of glycoproteins and phospholipids, albeit with possible gastrointestinal reactions such as abdominal distension and diarrhea. Rebamipide belonged to antioxidant, which contributed to gastric mucosa repair by increasing SOD and enhancing glutathione content [5]; nevertheless, undesirable reactions such as abnormal liver function and allergy would probably occur.

Increasing gastric mucosal protective agents were being used clinically, but their chemical-based agents would be hazardous to human body when taken for a

long period, thereby there was an urgent requirement to seek highly active green healthy gastric protective raw materials and develop innovative gastric protective products. Hitherto, multiple bioactive effects of protein peptides had been investigated and it was indicated that protein peptides had excellent intestinal absorption properties and outstanding physiological activity, which could have a preventive protective effect against alcohol acute gastric mucosal injury by safeguarding gastric mucosal defense barriers as well as elevating the level of anti-alcohol-induced oxidative free radicals [6, 7]. Soybean peptides treatment were revealed to substantially reduce ulcer index in mice, indicating its protective effect on the gastric mucosa of mice. Hydrolyzed wheat protein peptide exhibited beneficial effects on acute alcoholic gastric mucosal injury in rats, with mechanisms probably related to enhancing gastric mucosal protective factors, antioxidant, anti-inflammatory and anti-apoptotic effects [8]. Walnut oligopeptide possessed superior protective effect on gastric mucosal injury induced by anhydrous ethanol in rats, while its main mechanisms of effect were associated with reducing the direct damage of ethanol on gastric mucosa and promoting the synthesis of mucin [9]. Homemade cod skin protein peptides were initially investigated and debated for its pre-protective effect on ethanol-induced acute gastric mucosal injury in mice. In conclusion, usage of protein peptides to prevent and protect gastric mucosal injury became a critical theme.

Pneumatophorus japonicus was one of the important pelagic economic fishes in East China sea with wide distribution, fast growth, high yield and high nutritional value, which was beloved by people [10, 11]. The flesh of *Pneumatophorus japonicus* could be used as medicine for nourishing and strengthening, and could be therapeutic for chronic gastrointestinal diseases, tuberculosis and other injuries [12]. Researchers discovered that fish processing by-product wastes were abundant in protein and that their protein hydrolysates had additional advantages of being more digestible and easier to absorb, hence utilizing fish waste as a source of high value compounds was an excellent cost recovery strategy [13-15]. Gastric mucosal epithelial cells (GES-1) were derived from fetal gastric mucosal epithelium, and were immortalized

cells that were stably passaged in vitro. Since its establishment in 1992, it had been proven to be a good and practical experimental cell system. Therefore, GES-1 were selected as the cell model for in vitro experiments, which could be closer to clinical experiments at the molecular level [16]. Infrared spectroscopy and its microscopic imaging integrated image and spectroscopy technology, realizing simultaneous acquisition of spatial and spectral information of the sample [17], with technologies that permitted study the microscopic physical texture morphology (visible microscopic images) and chemical composition distribution characteristics (chemical microscopic images) of GES-1 under different processing conditions, construction of tissue or cell structure images through various algorithms which could objectively reflect the underlying health of samples [18], and the label-free, non-destructive chemical analysis provided was a tremendously attractive and potentially powerful tool for studying biological cells [19].

In this study, *Pneumatophorus japonicus* heads peptides (PHPs) were self-developed by enzymatic hydrolysis, and the protective effect of different molecular weights (<1 kDa, 1-3 kDa, 3-10 kDa), different concentrations of PHPs and positive drug gastric mucin on alcohol-damaged GES-1 were comprehensively investigated. Based on in-situ analysis techniques such as macro-, micro-, and nano-level molecular spectroscopy and microscopic imaging, supplemented by cellular biological methods, the dynamic regulation of the structure and composition distribution of alcohol-damaged GES-1 by various PHPs was systematically investigated. In situ visualization revealed the self-assembly regulations and component interactions of PHPs in GES-1 and subsequently the repair mechanism of PHPs on damaged GES-1, was explored. Furthermore, this study would offer theoretical guidance and technical reference for the future development of highly active gastric repair peptide products without side effects, and thus promote the high-quality utilization of aquatic waste products to protect our planet.

2. METHOD

2.1. Materials and Reagents

Fresh fish (*Pneumatophorus japonicus*) heads, were provided by Zhejiang Xingye Group Co., Ltd., China.

Two proteases (trypsin, alcalase) were provided by Shanghai Yuanye Biological Technology Co., Ltd. Both 10 kDa and 3 kDa ultrafiltration membranes were purchased from Millipore, 1 kDa ultrafiltration membranes were purchased from PALL. The Gastric Mucin was purchased from Beijing Jinming Biotechnology Co., Ltd. MTT Cell Proliferation and Cytotoxicity Assay Kit were provided by Shanghai Yuanye Biological Technology Co., Ltd. Lactate dehydrogenase LDH activity quantitative determination kit was purchased from Beijing Applygen Technology Co., Ltd.

2.2. Preparation of *Pneumatophorus japonicus* heads peptides (PHPs)

An appropriate number of fish heads were thawed, cleaned, drained crushed, and placed in a 500 mL conical flask, then, according to the best results of the laboratory's early single factor test, shaken in a water bath for hydrolysis. The solid-liquid ratio was 1:4 (W/V), the amount of enzyme added was 0.32% (Alkaline protease and trypsin enzyme activity ratio was 1:3), the enzymatic hydrolysis time was 4.03 h, and the enzymatic hydrolysis temperature was 50.95°C, after enzymatic hydrolysis was completed, the enzyme was inactivated at a constant temperature of 90°C water bath 15 min. The enzymatic solution was cooled to room temperature, and then centrifuged in a centrifuge for 10 minutes at 10,000 rpm/min. The supernatant was collected and filtered to obtain PHPs. Finally, PHPs were separated step by step through ultrafiltration membranes with molecular weight cutoffs of 10 kDa, 3 kDa, and 1 kDa, and PHPs of different molecular weights were collected and freeze-dried for 48 h before use.

2.2.1. Cell culture

Human gastric mucosal epithelial cells (GES-1) were purchased from Feng Hui Biotechnology Limited Company (Hunan, China). The cells were maintained RPMI-1640 complete medium (Gibco Company Inc., USA) supplemented with 10% Fetal Bovine Serum (FBS) (TIANHANG Biotechnology Company Inc., China) and 100 U. mL⁻¹ penicillin, 100 U. mL⁻¹ streptomycin in a humidified incubator with 5% CO₂ and 37°C. Cells were collected for use after entering the logarithmic growth phase.

2.2.2. Cell damage model exploration

GES-1 in logarithmic growth phase were digested and counted, then seeded in 96-well cell culture plate with 1×10^4 cells per well, and cultured in a humidified incubator at 5% CO₂ and 37 °C for 24 h until cells were essentially attached. GES-1 were incubated in medium containing alcohol (0, 2%, 4%, 6%, 8% and 10%) for 2 h, 3 h, 4 h, 5 h and 6 h respectively, following which MTT was performed. The test parameters at a cell survival inhibition rate of approximately 40% could be used as the best GES-1 injury model [20]. Set up 5 replicate wells for each dilution, and set up a blank control group at the same time.

2.2.3. Cell grouping and treatment

GES-1 were divided into normal group, model group, positive drug gastric mucin group (0.05 mg/mL) and PHPs group (Table S1). Among them, the PHPs group was divided into six groups with three molecular weight ranges of high concentration group (0.3 mg/mL) and low concentration group (0.06 mg/mL). GES-1 in logarithmic growth phase were digested and counted, then inoculated in 96-well cell culture plates with 1×10^4 cells per well, 100 μ L per well, placed in 37 °C, 5% CO₂ incubator until the cells were basically stick to the wall, draw out the culture medium in the well carefully with a syringe and discarded. The positive drug gastric mucin and PHPs medium with different molecular weight segments were added before damage (BD) and after damage (AD) induced by alcohol, 100 μ L per well, and the normal group and model group were not added, and cultured for 24 h. Each group was set up with 5 replicate wells, the edge wells of the culture plate were filled with sterile PBS, and a blank control group (without cells) was set.

2.2.4. MTT assay

The cells were treated as above in Cell Grouping and Treatment, and then performed the MTT test. Removed the old culture medium, replaced with 100 μ L fresh culture medium, added 10 μ L of MTT working solution (5 mg/mL) to each well, mixed at low speed and incubated in 37°C incubator for 4 h, then, aspirated the culture medium, added 100 μ L per well formazan dissolving solution, mixed well, put into incubator and continued to incubate for 4 h. After incubation, placed it on the microplate reader and mixed

well, and measured the absorbance at 570 nm [21]. Recorded the result, adjusted the colorimetric to zero with blank, and calculated the cell survival rate.

2.2.5. LDH cytotoxicity assay

Cell processing was the same as in Cell Grouping and Treatment above. Old culture medium was collected simultaneously during the MTT experiment. Determining the LDH activity of the medium according to the 2,4-dinitrophenylhydrazine method, the result was expressed as U [22].

2.2.6. Mitochondrial membrane potential determination

Analysis of mitochondrial membrane potential with fluorescent dye JC-1 purchased in Beyotime Biotechnology (Beijing China). When the mitochondrial membrane potential was high, JC-1 aggregated in the mitochondrial matrix to form J-aggregates, producing red fluorescence; when the mitochondrial membrane potential was low, JC-1 was incapable of aggregate in the mitochondria at this time, JC-1 was a monomer and produced green fluorescence. Consequently, it was extremely convenient to detect the change of mitochondrial membrane potential through the transition of fluorescence color. Preparation of the sample according to the requirements of the kit, and photographed with Laser Scanning Confocal Microscope.

2.2.7. Acquisition of infrared transmission imaging

Took 10 μ L of single cell suspension sample and dropped on CaF₂ sheet (13 mm x 2 mm) at room temperature (25°C, air humidity less than 40%) and dried for 2 h. After the sample was dried, the CaF₂ sheet was placed on the transmission imaging accessory stage of the infrared microscope imaging system, and an infrared light absorption map (100 μ m x 100 μ m) was collected after finding a suitable area in the view of the optical microscope. Infrared microscope images of the cell samples were acquired by Spectrum Image software with a spectral resolution of 4 cm⁻¹, an image pixel size of 6.25 μ m, 16 scans per pixel, and a wave number range of 4000-900 cm⁻¹ for the acquired spectra. Infrared microscope images of the cell samples were collected and analyzed by Spectrum Image software. After removing atmospheric noise and baseline correction, analysis was performed.

2.2.8. Acquisition of IR spectra

Fourier transform infrared spectrometer (PerkinElmer, Inc., USA), equipped with DTGS detector and ATR accessories.

Took 10 μL of GES-1 single cell suspension, dropped it on the center of CaF_2 sheet (12 mm x 1 mm), and dried it in a sealed container at room temperature (25°C, air humidity less than 40%) for 2 h, and placed it in a sealed and dry container to prevent the interference of moisture in the air before measuring the spectra. The IR spectra of the samples were detected with air as the background to eliminate the interfering effect of H_2O and CO_2 on the IR spectra of the samples. Then the spectra were collected by 32 scans in reflection mode, in the range of 4000-650 cm^{-1} with a resolution of 4 cm^{-1} .

2.2.9. Acquisition of second derivative IR spectra (SD-IR spectra)

SD-IR spectra were obtained after baseline correction and 13-point Savitsky Golay smoothing of average IR spectra using Spectrum10™ software (Version 10.6.0, PerkinElmer, Inc., USA).

2.3. Statistical Analysis

Experimental data were expressed as mean \pm standard deviation, and analyzed by ANOVA using SPSS 22.0 with p-value less than 0.05. Spectrum fitting was done by PeakFit v4. The related pictures were completed by Origin 2018.

3. RESULTS

3.1. Optimization of Cells Alcoholic Injury Model

The absorbance values of GES-1 cells at 570 nm responded to the cell damage by alcohol (Table S2). Various alcohol concentrations and treatment times inflicted distinct degrees of damage to GES-1. Among them, 8% alcohol incubation for 3 h had an inhibition rate of 40%, and this parametric equivalent of GES-1 was the best model of cell damage.

3.2. PHPs Protected GES-1 from Cell Viability Loss Induced by Alcohol

Compared with the model group, cell survival was increased in the GM and PHPs groups, indicating that GM and PHPs had a protective effect on damaged

GES-1 cells. PHP2H and PHP3H groups had similar protective effects to GM group, and both were superior to the other PHP groups (Figure 1).

When cell membrane ruptured due to cell damage, lactate dehydrogenase (LDH, a special protein in the cytoplasm of normal cells) was released, so the leakage of LDH corresponded with the degree of cell damage caused by drug treatment or other factors. After GES-1 were damaged by 8% alcohol, LDH leakage was dramatically increased, while the leakage was remarkably decreased in GM group resulting from the obvious reduction of apoptosis; after treating normal and damaged cells with varied concentrations of PHPs, LDH leakage in each group was drastically diminished, manifesting that PHPs had protection and repair effects on GES-1 (Table 1).

JC-1 probe was used to measure mitochondrial membrane potential and to investigate the effect of PHPs on mitochondrial function via red-green fluorescence ratio quantification [23]. Relative stabilization of mitochondrial membrane potential reflected the normal physiological activity of cells, and its long-term alteration could lead to modification of cellular activity [24]. Furthermore, researchers discovered that changes in mitochondrial membrane potential were associated with intracellular ATP production, oxidative stress status, etc [25]. Accordingly, a lower mitochondrial membrane potential was a hallmark phenomenon of cell death [26]. As shown in the normal cells, red-polarized mitochondria and green-depolarized mitochondria were both detected with dominant red fluorescence (Figure 2), which represented the mitochondrial membrane potential under physiological conditions. Red fluorescence was evidently weakened and green fluorescence was enhanced under alcohol treatment, indicating that treatment with alcohol induced an increment in mitochondrial membrane permeability and forfeited electronegativity, which affected cellular activity [27]; GM group and PHPs groups both achieved successful reversal of this change with a repairing effect on the cells. PHP3H group had the best positive effect, whether treatment was done by BD or AD.

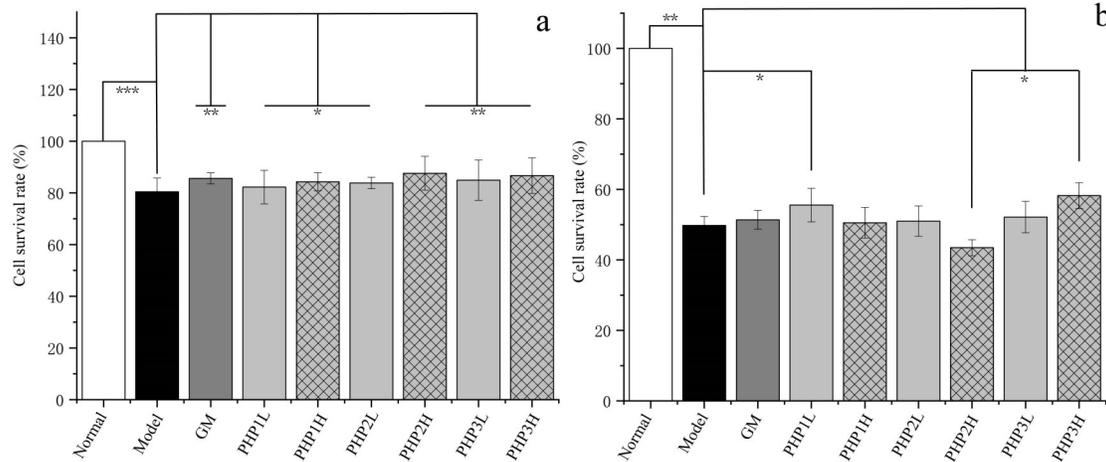


Figure 1. Cell survival rate under different treatment conditions. (a) Cells were treated through BD; (b) Cells were treated through AD.

Table 1. Lactate dehydrogenase (LDH) leakage from GES-1 cells

LDH leakage (U)	N	M	GM	PHP1L	PHP1H	PHP2L	PHP2H	PHP3L	PHP3H
BD	898.96±104.2	1083.41±38	928.59±25.9	884.15±14.5	937.48±10.6	946.37±16.5	1051.56±25.5	1087.48±24.2	1082.3±24.3
AD	960.44±11.7	1123.78±10.2	888.22±1.9	959.33±8.8	973.78±1.9	982.67±10	951.56±13.9	918.22±3.8	892.67±12

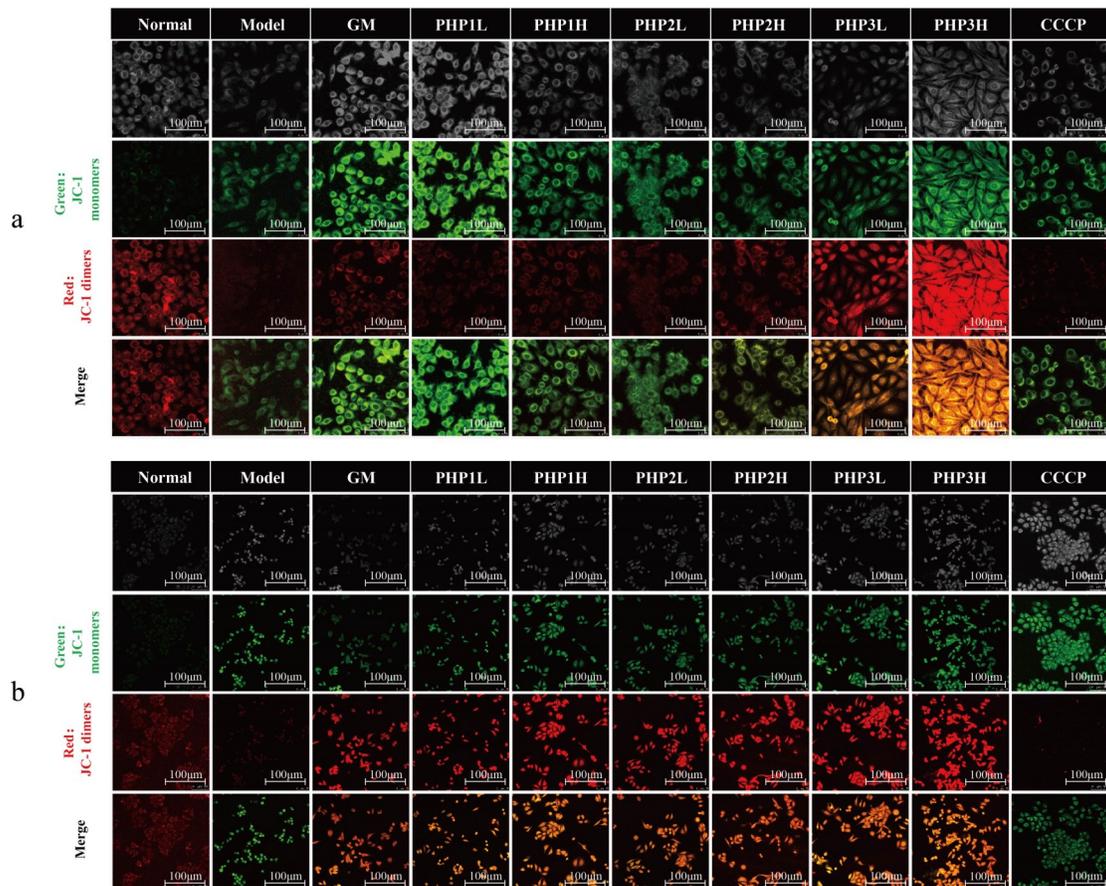


Figure 2. Laser scanning confocal image of JC-1. (a) Cells were treated through BD; (b) Cells were treated through AD.

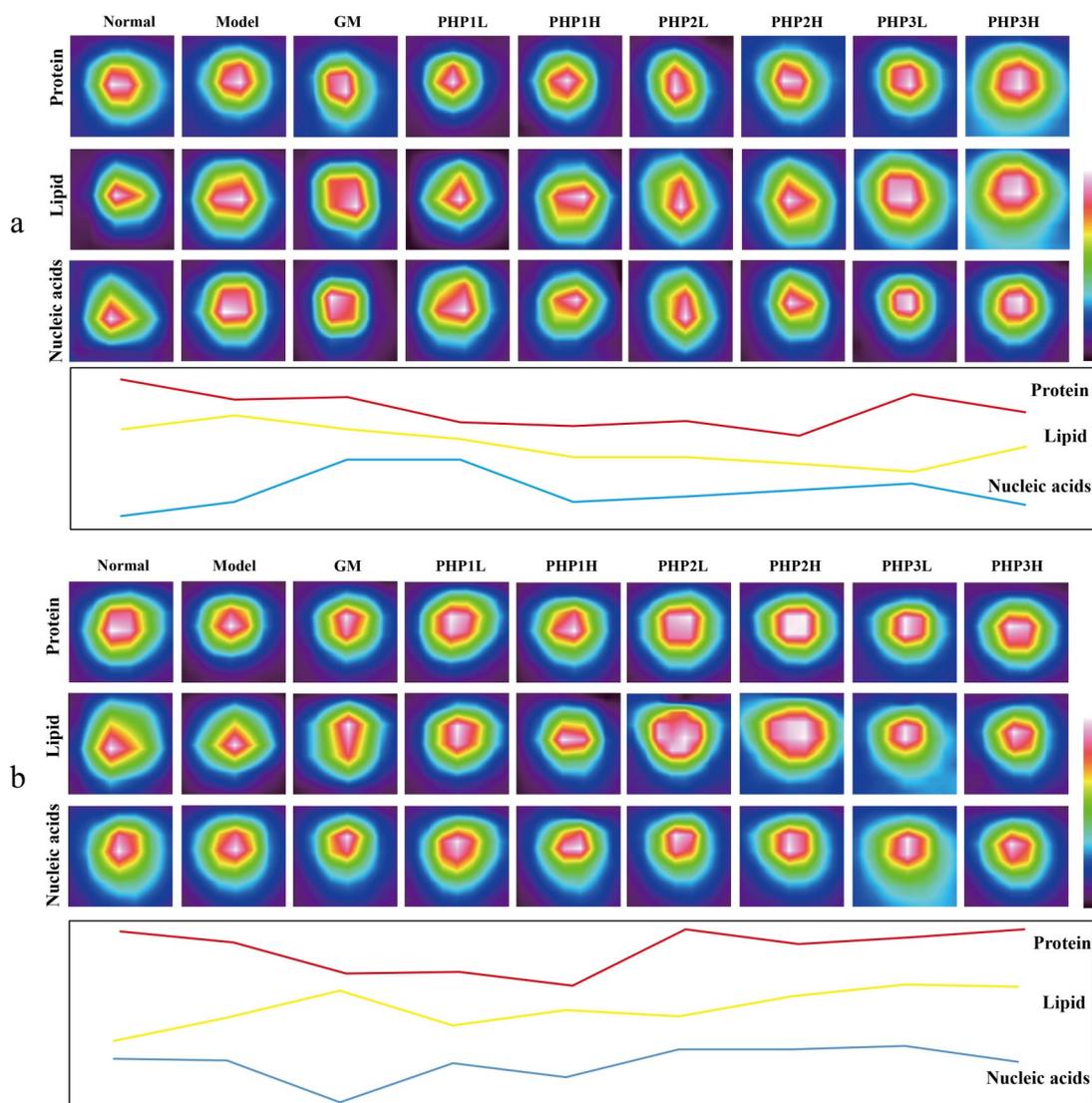


Figure 3. Infrared transmission imaging of cells under different treatment conditions. (a) Cells were treated through BD; (b) Cells were treated through AD.

3.3. Micro- and Macro-infrared Spectroscopic Analysis of Cellular Components under Diverse Treatments

3.3.1. Infrared transmission imaging of cells

Infrared transmission imaging was a fast, non-destructive, simple, and in-situ detection technology based on micron and submicron scales that was often used in complex mixture systems. Hundreds of vibrational spectra of samples were collected in specific areas, encompassing not only information on the spatial distribution of the samples in the micro area, but also information on the chemical composition of the samples, such as lipids, proteins and nucleic acids, etc. [28].

As the basic structural and functional unit of life activities, cells principally contained proteins, lipids and nucleic acids in terms of their material compositions. In consequence, according to infrared spectral transmission images, the wavenumber ranges of proteins ($1800\text{-}1500\text{ cm}^{-1}$), lipids ($1775\text{-}1735$, $1090\text{-}1075\text{ cm}^{-1}$) and nucleic acids ($1090\text{-}1075$, $980\text{-}950\text{ cm}^{-1}$) were extracted and their spatial distribution in GES-1 were analyzed. Normal cells damaged by alcohol demonstrated diminished protein percentage, enhanced lipid percentage, and enlarged nucleic acid percentage (Figure 3). Treatment with PHPs of various molecular weights resulted in varying percentages of proteins, lipids and nucleic acids.

3.3.2. FT-IR macroscopic fingerprinting analysis of GES-1 cells

To further explore the changes in the distribution of proteins, lipids, and nucleic acids inside cells, five spectra were extracted from each cell chemical image and the average spectrum was calculated. After different treatments, IR spectra of GES-1 cells showed distinctions in peak intensity and shape (Figure S1 & Table S3). Peak intensities and areas at 3000-2800 cm^{-1} (mainly attributed to fatty acids in the cell membrane) were increased in other treatment groups compared to the normal group. 1649 cm^{-1} (Amide I) and 1543 cm^{-1} (Amide II) peaks exhibited alterations in intensity and area under different treatment conditions. Spectra at 1200-900 cm^{-1} majorly contributed by polysaccharides and phosphate compounds (e.g., nucleic acids) in the cell wall showed a pronounced downward trend of peak intensity and area in the treated group compared with the normal group. Through the above analysis, macroscopic profiles of GES-1 under different treatment conditions could be illustrated. Nevertheless, the detailed changes in its lipids, proteins and carbohydrates were not available for comprehensive analysis. Hence, the second derivative spectra were performed on the peaks of different wavenumber bands and analyzed.

3.3.3. Effects of PHPs on intracellular lipids content

After early treatment with PHPs, lipids contents of cells were relatively increased after alcoholic injury, compared to the normal group (Figure 4A-a). The peak at 1740 cm^{-1} is mainly caused by the C=O stretching vibration of esters contained in phospholipids and fatty acids. The content of esters in the cell membrane was increased in all groups compared to the normal group of cells (Figure 4A-a). Among groups treated through AD, IR spectra of GES-1 in PHP1 groups indicated a noticeable increment in peak intensity at 1740 cm^{-1} , presumably attributed to small molecular weight range of the protein peptide itself contained a small amount of esters, which penetrated into damaged GES-1 cells after melting in medium, contributing to the evident variation in peak intensity (Figure 4A-b). Nevertheless, peak intensities of the PHP2 and PHP3 groups were slightly changed compared to normal cells with fluctuations inside a particular range (Figure 4A-b); it was

speculated that PHPs melting in the culture medium formed a protective film to prevent further cells from being further damaged and to protect undamaged cells due to their high molecular weight.

Since majority of lipids contained long hydrocarbon chains that capture changes in lipids, ratios based on the peak intensities of 2923 and 2956 cm^{-1} (A_{2923}/A_{2956}) were employed to analyze compositional changes in lipids [29]. In groups treatment through BD, ratios increased to a certain extent in all other groups compared to normal group, and PHP3L group had the highest ratio (Figure 4B-a). A_{2923}/A_{2956} changed to varying degrees with treatment of PHPs at diverse molecular weights among AD-treated groups (Figure 4B-b). Addition of A_{2923}/A_{2956} indicated that amount of methylene groups (CH_2) relative to methyl groups (CH_3) was increased in the lipids of the cells, implying an expansion of aliphatic hydrocarbon chains [30]. Inasmuch as lipids were predominantly present on cell membranes, growth of hydrocarbon chains could demonstrate that fluidity and permeability of membrane became enhanced following cell damage by alcohol, thereby altering the manner in which phospholipid bilayers fold. This is related to Phospholipid-mediated signaling pathways.

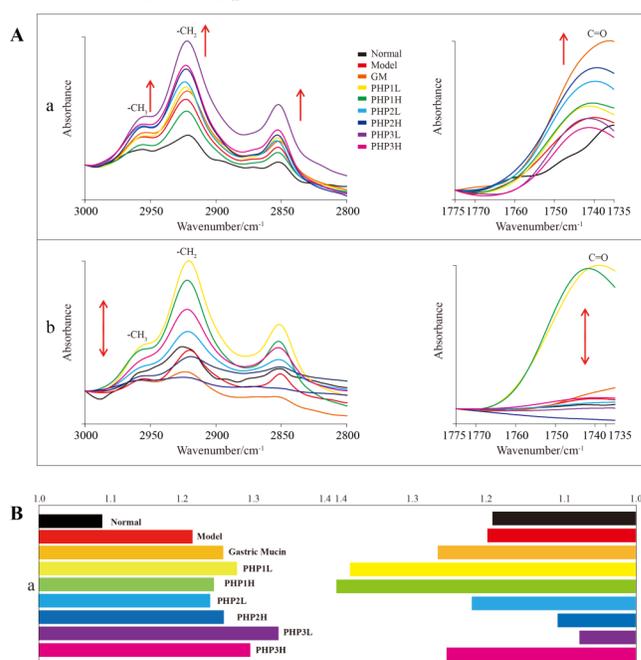


Figure 4. A: IR spectra of GES-1 cells at 3000-2800 cm^{-1} and 1775-1735 cm^{-1} ; B: The ratio between the peak intensities of GES-1 cells at 2923 and 2953 cm^{-1} . (a) Cells were treated through BD; (b) Cells were treated through AD.

3.3.4. Effects of PHPs on intracellular protein contents

1649 and 1544 cm^{-1} were respectively conferred with stretching vibration of C=O (Amide I) and N-H bending vibration (Amide II) in the protein backbone-peptide bond [31]. Compared with the normal group, protein content of cells damaged by alcohol diminished, and content of amide I and amide II showed a remarkable downward trend (Figure 5A). To further reveal the variation of intracellular protein content under different treatment conditions, second derivative spectra were performed at 1800-1400 cm^{-1} for all groups of original spectra to enhance their small differences. By separating the overlapping peaks in the one-dimensional spectra, small absorption peaks and overlapping peaks that were not visible in the original spectrum could be identified. Specific compound characteristic peaks enhanced fingerprinting of the spectra and clarified the analysis of slight discrepancies in similar spectra.

Infrared spectrogram fitting could be utilized to reveal changes in cellular protein secondary structure under different treatment conditions [32]. Spectral curve fitting was primarily based on Gaussian fitting, which performed peak-finding and iterative operations on selected characteristic bands (Figure S2). This method enabled the rapid separation of protein secondary structures from original spectra. Amide I of proteins were frequently quoted in FTIR to resolve changes in protein secondary structure. α -helix (1658~1650 cm^{-1}), β -turn (1695~1660 cm^{-1}), β -sheet (1640~1610 cm^{-1}) and random coil (1650~1640 cm^{-1}) were indicated as the four principal types of protein secondary structure, arising from the dipole moment coupling triggered by oscillation between carbonyl groups (Figure 5A). Protein secondary structures of GES-1 were interconverted throughout differing treatment conditions (Figure 5B). PHPs and gastric mucin treatment elevated the downward trend in α -helix and random coil contents attributable to alcohol damage that approached normal cells, while simultaneously calibrating upsurge the contents of β -sheet and β -turn caused in alcohol damage.

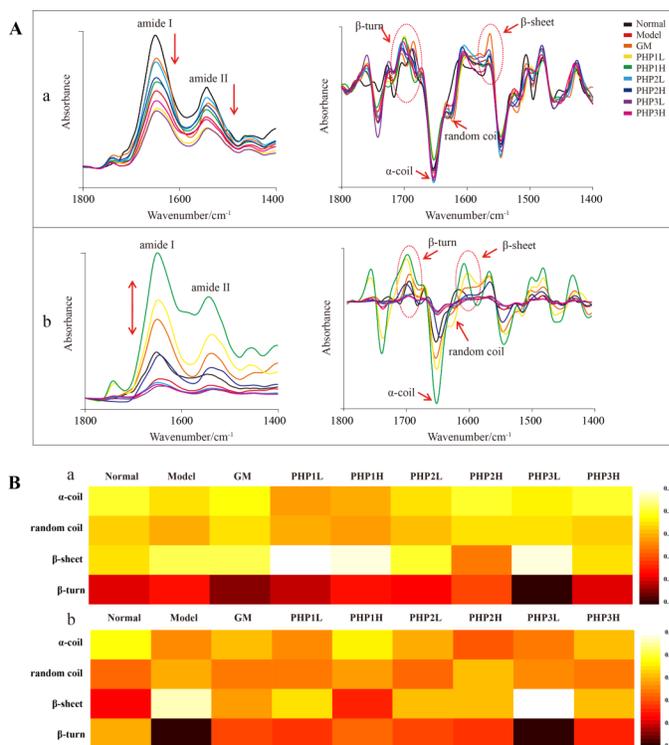


Figure 5. A: IR and SD-IR spectra of GES-1 cells at 1800-1400 cm^{-1} ; B: Heat map of cellular protein secondary structure content changes under different treatment conditions. (a) Cells were treated through BD; (b) Cells were treated through AD.

3.3.5. Effects of PHPs on intracellular carbohydrate contents

1200-900 cm^{-1} (carbohydrate region): contained information on polysaccharides within bacterial cell walls along with phosphate compounds such as nucleic acids in bacterial cell walls [33]. Among groups treatment through AD, 968 cm^{-1} (a stretching vibration of C-C/C-O in the DNA backbone of deoxyribose and phosphodiester bonds) shifted towards the higher wave number (blue shift), whilst 1086 cm^{-1} (a symmetric stretching vibration of the phosphodiester group PO_2^-) demonstrated a shift towards the lower wave number (red shift) (Figure 6a). This indicated that a structural alteration of the nucleic acid molecules and an impact on the internal hydrogen bonding forces engaged in GES-1 after treatment. After alcoholic damage, intracellular polysaccharide contents depreciated, amongst which PHP3H groups were approximated to normal cells (Figure 6).

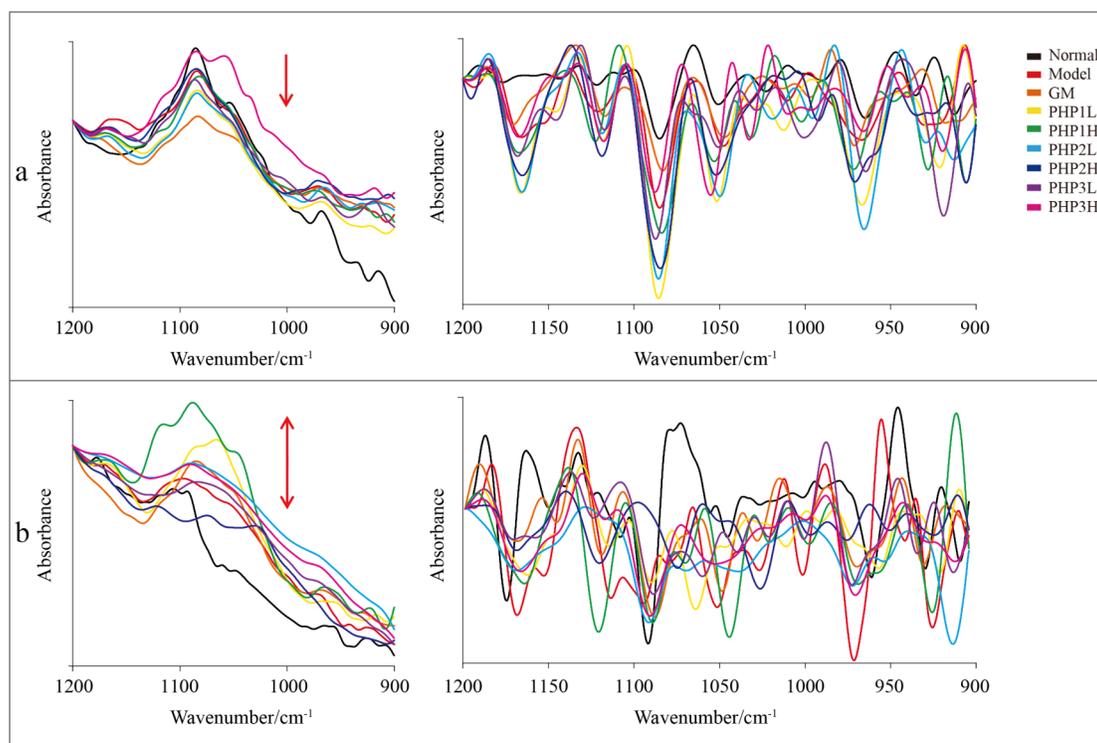


Figure 6. IR and SD-IR spectra of GES-1 cells at 1200-900 cm^{-1} . (a) Cells were treated through BD; (b) Cells were treated through AD.

4. DISCUSSION

Excessive alcohol consumption exposed high concentrations of ethanol to gastric tissues, leading to gastric mucosal injury by triggering oxidative stress and inflammatory signaling pathways and then stimulating epithelial cell apoptosis [34]. It was revealed that, after an 8% alcohol injury in normal cells for 3h, GES-1 activity declined, and intracellular protein substances decreased, while lipid substances and nucleic acid substances increased. In contrast, GES-1 treated with PHPs manifestly promoted cell proliferation, minimized LDH release and augmented mitochondrial membrane potential. In particular, protection and repair effects of PHP3 groups were optimal.

Peptides were hydrolyzed products of proteins and had superior absorption properties than macromolecular proteins. This research investigated the protective effects of differing molecular weights and concentrations of PHPs on GES-1, and compared them with the positive drug gastric mucin. The results indicated that PHP3 groups outperformed the other groups. This presumably could be attributed to more

abundant amino acids in the high molecular weight segment of PHPs. Due to their higher molecular weight, they formed a protective film that protected damaged cells from further deterioration and prevented undamaged cells from being disrupted, thus providing a relatively effective supplementary support role.

The gel formed by the overlapping of glycoprotein molecules was an essential component of gastric mucus, which formed a gastric mucus-bicarbonate barrier with bicarbonate. Meanwhile, the lack of glutathione (GSH) in the mucosa potentially permitted accumulation of free radicals, exacerbated oxidative stress and damaged gastric mucosa [35]. According to the "macro-micro-nano" multi-level molecular spectroscopic analysis, incorporation of PHPs enhanced contents of definite proteins in cells, and high-molecular-weight fragments of PHPs distinctly promoted the synthesis of glycoproteins and modified quality of gastric mucosal mucus layer, whereupon it reduced the damage of mucosal structure and the production of inflammatory factors by alcohol, thus playing a

protective and repairing role.

Alcohol intake facilitated oxidative stress in tissues, leading to augmentation of lipid peroxidation, i.e., ROS interacted with macromolecular substances such as phospholipids, enzymes and membrane receptors of biological membranes, polyunsaturated fatty acid side chains and nucleic acids to form lipid peroxidation products, which altered the fluidity and permeability of cell membranes, contributing to alterations in cell structure and function, and ultimately culminating in gastric damage. At the identical time, damaged mitochondria also generated ROS, which evoked a rapid depolarization of the inner mitochondrial membrane potential [36]. Consequently, antioxidant activity could be used as an indicator to evaluate the resistance and repair capacity of body and gastric tissue against gastric mucosal damage [37]. Additionally, ROS generated by ethanol suppressed expression of Hsp70 protein while enhancing expression of Bax protein. Hsp70 refolded denatured proteins and repaired proteins to protect cells from damage [38]. Increased Bax protein promoted apoptosis [39]. Accordingly, gastric protection could be accomplished by inhibiting the production of ROS. Experimental results of IR imaging and IR spectroscopy indicated that alterations in intracellular lipids, nucleic acids and proteins were effectively reversed by either BD or AD treatment. Hypothetically, PHPs addition blocked the ROS signaling pathway, enabling an increment in SOD, converting ROS into hydrogen peroxide, which in turn was converted to water via GSH peroxidase in mitochondria, thereby interfering with the initiation of apoptotic program and subsequently protecting gastric mucosa.

In summary, PHP3 groups apparently reversed alcohol-induced damage in GES-1 by improving cell viability, reducing LDH liberation, increasing mitochondrial membrane potential, and reversing proteins, lipids and nucleic acids contents in cells. Henceforth, further exploration of signaling pathways as well as further isolation and purification of PHPs were warranted to ascertain their efficacy (Figure S3).

5. CONCLUSION

It was demonstrated that self-developed PHPs could

alleviate alcohol-induced damage to GES-1. PHPs dramatically reduced LDH leakage, boosted cell proliferation and heightened mitochondrial membrane potential. Specifically, PHP3H group blocked the ROS signaling pathway to promote synthesis of glycoproteins, increase SOD, inhibit production of ROS and interfere with the apoptotic program, thus protecting gastric mucosa. The structure and sequence of this peptide would be demonstrated in subsequent experiments; moreover, further studies were required to determine whether the same effect was observed in other parts of *Pneumatophorus japonicus*. Altogether, PHPs were favorable green natural preparations that could be further exploited to be green medicines or healthcare products for the treatment of alcohol-induced gastric mucosal damage, thereupon implementing a meaningful development approach of aquatic waste from low-value products to high-value products, solving critical social problems such as resource waste and environmental pollution.

Acknowledgements

This work was financially supported by the National Key R&D Program of China (Grant No.: 2020YFD0900905).

Conflicts of interest

There are no conflicts to declare.

Supplementary material

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