Gastroprotective effects of Nelumbinis Rhizomatis Nodus carbonisata-derived carbon dots on ethanol-induced gastric ulcers in rats

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Research

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Abstract

Background

Gastric ulcers is a common gastrointestinal digestive system disease. Considering the frequency of human gastric ulcers, the side effects and cost of some existing synthetic drugs, the use of natural products is an important choice for many people. The aim of present study was to explore gastroprotective effects of nelumbinis rhizomatis nodus carbonisata carbon dots (NRNC-CDs) on ethanol-induced gastric ulcers in rats.

Methods

The NRNC-CDs were synthesized via high temperature calcinations treatment at 350 °C for 1 h were characterized by various spectroscopic and electron microscopy techniques for their structural, morphological, and optical properties. In vitro cytotoxicity of CDs for the human gastric epithelial cells line (GES-1 cells) was assessed by the CCK-8 assay. Furthermore, the study evaluated gastroprotective effects of NRNC-CDs on ethanol-induced gastric ulcers in rats, followed by a preliminary study on the possible mechanisms of gastroprotection.

Resultes

NRNC-CDs with a quantum yield of 1.38% have an average diameter of 2.89±0.82nm and the lattice spacing of 0.29 nm , and exerted low toxicity to GES-1 cells by CCK-8 test. In vivo experiments showed that NRNC-CDs remarkably reduced gastric mucosal damage and significantly increased the levels of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GSH-Px). In addition, NRNC-CDs also significantly inhibited tumor necrosis factor-alpha (TNF-α) and pro-inflammatory interleukin-6 (IL-6) level in gastric tissues. Histological findings demonstrated that NRNC-CDs exhibited a protective effect against tissue alterations in response to the ethanol-induced ulcer.

Conclussion

The potent gastroprotective effect of NRNC-CDs were thus attributed to its anti-inflammatory and antioxidant effects. This discovery provides guidance for further research the effect of CDs in gastrointestinal digestive diseases.

Background

Gastric ulcers is a common gastrointestinal digestive system disease, which occurs owing to an imbalance between the defensive function and aggressive factors of the mucosal barrier[1]. These aggressive factors mainly include physical stress, heavy tobacco inhalation, excessive alcohol or caffeine, non-steroidal anti-inflammatory drugs, and microorganism infection[2]. Especially ethanol, the common factor existed in our daily life, plays a vital roles in the development of gastric cell necrosis and vascular injury, which was further induce gastric damage and disease[3]. At present, clinical strategies for
management of treating gastric ulcer is to use proton pump inhibitors and H₂ receptor antagonists, which achieved an effective ulcer healing process by inhibiting gastric acid secretion, neutralize gastric acid and inhibit cell apoptosis through cytoprotection[4, 5]. However, the use of these drugs are cause some side effects, such as arrhythmias, nausea, constipation and hypergastrinemia, etc[5, 6]. Accordingly, it is necessary to develop novel anti-gastric ulcer products, as a promising therapeutic resource, which may emerge fewer side effects.

Carbon dots (CDs) are a new class of nanoscale materials with abundant surface functional groups (amino, carbonyl, carboxyl, or hydroxyl), and have attracted tremendous attention because of their possessed multiple considerable benefits such as photochemical stability, ease of functionalization, high biocompatibility, hypotoxicity and hydrophilicity, etc.[7, 8]. Moreover, it have been reported that administration of CDs have no significant effect on blood biochemistry and hematology analysis in vivo, and caused no histopathological abnormalities on major organs[9]. Although the drug is administered in different ways, the CDs could be excreted from the body efficiently and rapidly[10]. Thus, these unique properties make CDs a promising candidate in the clinical applications[11-13].

Nelumbinis Rhizomatis Nodus (NRN), known as “Oujie” in Chinese, is originated from the dried rhizome node part of Nelumbo nucifera Gaertn. NRN is often made into a charcoal processed product for use, as name Nelumbinis Rhizomatis Nodus carbonisata (NRNC). Herein, in the study, Nelumbinis Rhizomatis Nodus carbonisata as carbon source were treated to prepare the CDs by a simple, eco-friendly method, which was named the Nelumbinis Rhizomatis Nodus carbonisata carbon dots(NRNC-CDs). Furthermore, the study assessed the protection effects of NRNC-CDs on ethanol-induced gastric ulcer in rat. Accordingly, Some histopathological damage and biochemical indexes were evaluated.

Results

Characterization of the NRNC-CDs

The TEM image (Fig. 1A) clearly indicates that the formed NRNC-CDs were spherical morphology with homogeneous dispersion. The particle size distribution calculated from hundred particles of CDs reveal that narrowly distributed CDs have an average diameter of 2.89±0.82nm. The HRTEM image (Fig.1B and 1C) were shows that NRNC-CDs revealed the lattice spacing of 0.29 nm. The XRD patterns of NRNC-CDs presented the broad diffraction peak at 22.0° associated with the formation of amorphous graphitic carbon in the Fig.1D, which was consistent with the TEM results.

The spectral properties of NRNC-CDs were shown in Fig.2A. The UV-Vis absorption band was observed at 270 nm, which was contributed to the π−π⁺ transition of C=C on the surface of CDs[14]. The excitation and emission fluorescence spectrum of NRNC-CDs indicated that the maximum excitation exhibited at 354nm and the maximum emission at 456 nm. The quantum yield (QY) of NRNC-CDs was calculated to be 1.28%. The surface functional groups of NRNC-CDs were analyzed by the FTIR spectrum, as shown in Fig.2B, the peak was corresponded at 3441 cm⁻¹, which resulted from the stretching vibration of O-H or
N-H. The strong characteristic absorption peaks at 2932 cm\(^{-1}\) corresponds to the existence of the CH\(_2\)-stretch. The absorption peaks at 1629 cm\(^{-1}\) and 1400 cm\(^{-1}\) were assigned to C=O and C-N, respectively. The peaks at 1047 cm\(^{-1}\) corresponded to the symmetric stretching vibrations of C-O-C[15, 16].

The XPS was employed to evaluate the surface composition of NRNC-CDs. The XPS full scan spectrum of NRNC-CDs (Fig.2C) displayed that the NRNC-CDs were mainly composed by C, O and N elements, which are attributed to 70.21%, 23.28% and 3.60%, respectively. The C1s XPS spectrum of NRNC-CDs (Fig.2D) was could be divided into two peaks centered at 283.90eV (C-C) and 285.20eV (C-N).The O1s XPS spectrum of NRNC-CDs (Fig.2E) displayed three major subpeaks at 530.60eV (C=O) and 531.60eV (C-O-C).The high resolution N1s spectrum (Fig.2F) presented two major subpeaks at 398.95eV (C-N) and 400.10eV (N-H)[17].

**Cell viability assay**

As shown in Fig.3, the CCK-8 study revealed that the average cell viability was greater than 95% at a concentration of NRNC-CDs up to 500 \(\mu\)g/mL. The cell viability was higher than 100% when NRNC-CDs concentration was below 500 \(\mu\)g/mL. Cell viability gradually decreased as the NRNC-CDs concentration increased from 500 to 1000 \(\mu\)g/mL, which exerted low toxicity to GES-1 cells at a relatively high concentration.

**Macroscopic evaluation of gastric lesions**

The gross appearance of the rat stomachs after treatment with ethanol to induce gastric mucosal damage(Fig.4A). There was no congestion, edema, erosion or ulceration in the control group, but the model group rats showed severe damage in the gastric mucosa as evidenced by ulcerated and hemorrhagic lesions. As shown in Fig.4B, the model group presented severe mucosal injury with an average ulcer index (15.34±3.63)%. Compared with the model group, ranitidine and NRNC-CDs (5.00, 2.50 and 1.25 mg/kg) significantly decreased ulcer index by (3.03±1.96)%, (2.19±1.47)%, (5.47±1.99)% and (8.20±2.63)%(\(P<0.01\)), respectively. Moreover, oral administration of ranitidine and NRNC-CDs at doses of high, medium and low ameliorated damage to the gastric mucosa by 80.21%, 85.73%, 64.36% and 46.52% (\(P<0.01\)), respectively(Fig.4C).

**Effect of NRNC-CDs on antioxidant and anti-inflammatory activity in the gastric tissue homogenate**

The Fig.5 showed that the effect of NRNC-CDs on antioxidant and anti-inflammatory activity in the gastric tissue homogenate. The SOD, CAT, GSH and GSH-Px activities in the gastric tissue homogenate of the model group were significantly lower than those in the control group (\(P<0.05\)). As shown in Fig.5A, administration of NRNC-CDs at the doses of high and medium, similar to ranitidine group, exhibited a significant increase in SOD level in stomach tissue (\(P<0.05\)). Administration of NRNC-CDs and ranitidine increased gastric CAT level, compared with the model group(Fig.5B). The activities of GSH-Px and GSH in the high-, medium- and low doses of NRNC-CDs (high and medium) treatment group were significantly higher than those in the model group (\(P<0.05\)) in the Fig.5C and 5D. Although the low dose did not
achieve statistical significance, increase in SOD, GSH-Px and GSH productions were also observed compared with the model group.

As shown in Fig.5E and 5F, there was a respectively high level of TNF-α and IL-6 in the model group as compared with the control group ($P < 0.01$). However, NRNC-CDs at high, medium and low dose significantly declined the levels of the pro-inflammatory cytokines including TNF-α and IL-6 ($P < 0.01$).

**Histopathological examination**

Histological analyses of the gastric mucosa were depicted in Fig. 6. The microscopic study of the control group (Fig.6A,a) shows typical gastric histoarchitecture with intact epithelium and glands. Ethanol administration caused extensive damage of gastric mucosa characterized by hyperemia, severe desquamation and loss of surface epithelial(Fig.6B,b). Pre-treatment with ranitidine (Fig. 6C,c) and NRNC-CDs(Fig. 6D-F,d-f) for 7 days had comparatively better protection of the gastric mucosa as seen by reduction in ulcer area, reduced or absence of hemorrhage and desquamation.Interestingly, treatment with NRNC-CDs significantly reduced the degeneration and hemorrhage induced by ethanol, indicating that protective action that was evident with the high dose of 5 mg/kg.

**Discussion**

The CDs have many fascinating properties and excellent biocompatibility with size of less than 10 nm[18]. The development of a green, simple and eco-friendly method for the preparation of bio-sourced CDs has become the focus of research with the development and in-depth study of CDs. It has been reported that many natural plants as precursors were prepared the green CDs, such as pear juice [19] ,gynostemma [20],wheat bran [21] and citrus fruit peels [22], etc. In this paper, the novel NRNC-CDs were prepared from NRN by a simple method, and this method no added any toxic reagents or chemicals. These CDs are required no further tedious modification and purification steps [23-25]. In recent years, CDs are widely used in biomedical sciences, and the most studied property of CDs are their photoluminescence owing to its wide application in bioimaging, optoelectronics and spectroscopic methods[26]. With continuous in-depth research on carbon dots, it has been found that it has great potential in treating diseases[27]. It was found in previous studies that pollen typhae carbonisata-derived CDs proved to be an effective substance for hemostasis[14], mulberry silkworm cocoon-derived CDs possess antiinflammatory[30] and fructus crataegi-derived CDs reduce postprandial blood glucose levels[31]. These CDs derived from different precursors, and they have different biological activities.

Considering the frequency of human gastric ulcers, the side effects and cost of some existing synthetic drugs, the use of natural products is an important choice for many people[28]. In the study, we were firstly studied the gastroprotective effect of NRNC-CDs in an experimental gastric injury model induced by ethanol. CDs were prepared from a green carbon sources(NRN). The choice of precursor plays a key role in the physical and chemical properties of carbon dots. NRN as a natural materials contain basic elements such as carbon, nitrogen, oxygen, sulfur and phosphorus, which provide unique surface functional groups and properties for CDs. Moreover, this plant is abundant resources and economic, and
it can be widely used in clinical. Besides, the NRNC-CDs exerted low toxicity to GES-1 cells by CCK-8 test. Accordingly, further comprehensive researches of CDs on gastric ulcer is significance meaningful.

Ethanol-induced gastric ulcer induced was a classic model, which is often used to screen substances with potential effects on gastric ulcer. As we all know, ethanol is considered to be a cause of gastric injury due to it changes protective factors, including reducing mucus production and blood circulation in the mucosa[29, 30]. Ethanol consumption can produce oxidative stress and acute inflammatory reaction in animals[31]. SOD, CAT and GSH constitute an endogenous antioxidant system, which can be used to remove excess oxygen-derived free radicals and maintain them at physiological levels[32]. GSH-Px also plays an important role in protecting against oxidative gastric mucosal injury[33]. In this experiments, we firstly showed that ethanol administration clearly altered the gastric mucosa by macroscopic evaluation of gastric lesions, and the NRNC-CDs significantly reduced the areas of gastric ulcer formation and ulcer index, suggesting the ability of these CDs in protecting gastric ulcer. In addition, we also showed in the present study that ethanol intoxication induced the depletion antioxidant enzyme activities such as SOD, CAT, GSH and GSH-Px. The stomach tissue of the body is full of blood vessels and has an adequate blood supply. When the stomach tissue is stimulated by foreign substances or conditioned, it can produce a large amount of oxygen free radicals. These substances can induce cell apoptosis and aggravate gastric mucosal damage[34]. The study results showed that NRNC-CDs remarkably reduced gastric mucosal damage and significantly increased the levels of SOD, CAT, GSH and GSH-Px, and that were reveal that the gastric homogenate of the NRNC-CDs pretreatment group potent gastric protection by alleviating oxidative stress.

The inflammation is an inevitable mediator in ethanol-induced gastric ulcers, which have accompanied the increased expression of pro-inflammatory cytokines, such as TNF-α and IL-6[35]. Moreover, TNF-α and IL-6 levels were associated with the severity of gastric mucosal inflammation. There increasing IL-6 and TNF-α levels in gastric tissue can be attributable to the necrotizing effects of ethanol in the model group. These results of our study showed that pretreatment and treatment with NRNC-CDs could remarkably alleviate inflammation symptoms by suppressing the production of TNF-α and IL-6, which was consistent with previous findings[36, 37].

In summary, this experiment demonstrated that the NRNC-CDs have important ulcer protection properties in a rat model of gastric ulcer induced by ethanol. In the study, it was proved that NRNC-CDs also has an gastric protection effect, and that were promoted ulcer healing by reducing oxidative stress and reducing the inflammatory response. Thus, NRNC-CDs may be developed a potential drug for the treatment of gastric ulcer.

Conclusions

In the work, a novel eco-friendly NRNC-CDs were prepar by a simple method, and their were found to be nontoxic toward GES-1 cells. Interestly, The results indicated that the NRNC-CDs were firstly proved the gastroprotective effects on ethanol-induced gastric ulcers in rats. The NRNC-CDs effectively reduced the
ulcer index of the gastric mucosa and increased the percentage of ulcer inhibition macroscopically. Furthermore, the levels of SOD, CAT, GSH and GSH-Px increased and the levels of TNF-α and IL-6 were significantly decreased, which can prove that this protection may be related to the antioxidant properties of NRNC-CDs by activating some enzymatic antioxidant mechanisms and attenuating the inflammatory response.

Materials And Methods

Materials

Dialysis membranes (molecular weight cut off was 1000 Da) were purchased from Beijing Ruida Henghui Technology Development Co., Ltd (Beijing, China). The cell counting kit (CCK)-8 was brought from Dojindo Molecular Technologies, Inc.(Kumamoto, Japan). The Dulbecco’s Modified Eagle Medium (DMEM) and the fetal bovine serum (FBS) were purchased from mediatech, Inc. (Conning, USA). Absolute ethanol (EtOH) solution was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GSH-Px) kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). The ELISA kits to measure tumor necrosis factor-α (TNF-α) were purchased from Proteintech Group, Inc (USA), and the ELISA kits to measure interleukin 6 (IL-6) were purchased from Abcam (Amyjet scientific inc, UK). Deionized water was used as a solvent for all experiments.

Animals

A total of 50 male Sprague-Dawley (SD) rats (180-200 g) were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Animals were acquired from the Laboratory Animal Centre, Beijing University of Chinese Medicine, and were bred in an environmentally controlled breeding room (temperature: 20-25°C) for 1 week prior to the start of the experiments and provided with standard laboratory food and water. Experimental protocols used in the present study were approved by the Animal Experimental Ethics Committee of Beijing University of Chinese Medicine (Beijing, China). All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

Preparation of carbon dots

CDs were prepared from NRN by using the modified pyrolysis method[38]. First, the NRN (80.0g) was placed in crucible, which was transferred into a muffle furnace (TL0612, Beijing Zhong Ke Aobo Technology Co., Ltd, China) and carbonized at 350°C for 1h, yielding NRNC. After allowing the temperature to drop naturally to 30°C, the NRNC was grinded to powder and boiled twice in distilled water at 100°C for 1h each time. The solution was cooled to room temperature and filtered with a 0.22μm filter membrane to remove any large particles, and that solutions was purified with a 1000 Da dialysis bag in deionized water for 7 days to remove the small molecules. Finally, the solution inside the dialysis membrane was collected for further characterization and usage.
Characterization of NRNC-CDs

The surface morphology of NRNC-CDs were obtained by transmission electron microscopy (TEM, Tecnai G2 F20 electron microscope). Structural details were obtained using a JEM-1230 high-resolution transmission electron microscopy (HRTEM; Japan Electron Optics Laboratory, Tokyo, Japan). The UV–vis absorption was determined using were recorded by spectroscopy (CECIL, Cambridge, UK) in the absorption wavelength 200-600 nm at 25°C. The fluorescence spectra (F-4500; Tokyo, Japan) were monitored with a molecular fluorescence spectrometer in a standard quartz cuvette. The functional groups of NRNC-CDs were recorded by performing Fourier transform infrared spectroscopy (FTIR) spectroscopy (Thermo Fisher Scientific, CA, USA). X-ray photoelectron spectroscopy (XPS) of the sample was recorded using an ESCALAB 250Xi XPS (Thermo Fisher Scientific) using a mono x-ray source Al Kα 150 W. The X-ray diffraction (XRD) patterns were characterized on an x-ray diffractometer (Bruker AXS, Karlsruhe, Germany) using Cu Kα radiation (λ=1.5418 Å).

Quantum yield measurement

Quantum yield (QY) was measured with quinine sulfate in 0.1 M H₂SO₄ (quantum yield 54%) as a standard sample. The QY of NRNC-CDs was estimated according to the follow equation:

\[ \Phi = \Phi_r \times \frac{I}{I_r} \times \frac{A_r}{A} \times \frac{\eta^2}{\eta_r^2} \]

Where the ‘Φ’ is the fluorescence quantum yield, ‘I’ represents the integrated fluorescent intensity and ‘A’ stands for the absorbance. The ‘η’ is referred as the refractive index of the solvent, and the subscript ‘r’ refer to quinine sulfate.

Cell viability assay

In vitro cytotoxicity of NRNC-CDs for the human gastric epithelial cells line (GES-1 cells) was assessed by the CCK-8 assay. The GES-1 cells cultured in supplemented with 15% FBS and 1% penicillin-streptomycin (Mediatech, Manassas, VA, US) incubated at 37°C under humidified 5% CO₂. Then, the culture medium was discarded and the GES-1 cells were incubated in the fresh culture medium containing NRNC-CDs with different doses of the NRNC-CDs solutions (1000, 500, 250, 125, 62.5, 31.25 μg/mL) for another 24 h, and DMEM was selected as the positive. Next, the cells attached to each plate were treated with 10μL CCK-8 solution for 4 h incubation. Finally, the microplate reader (Biotek, VT, USA) was use to read absorbance at 450 nm. The cell viability was calculated according to the following equation:

\[ \text{Cell viability(\%)} = \frac{A_{\text{CDs}}}{A_{\text{Control}}} \times 100\% \]
where \( A_{\text{CDs}} \) and \( A_{\text{control}} \) are the absorbance of cells incubated in the presence and absence of NRNC-CDs, respectively.

**Induction of acute gastric lesion by ethanol**

All animals were randomly divided into six groups of ten rats each for intragastric administration. The groups were divided into: control group, gastric ulcer groups (model group), ranitidine control (50.00 mg/kg), and the NRNC-CDs treatment groups (5, 2.5 and 1.25 mg/kg = high-dose, medium-dose and low-dose, respectively). All groups received prophylactic administration for 7 consecutive days. The rats were fasted within the 24 h ahead of the last administration. 2h after the final administration, acute gastric ulcer model was made by absolute ethanol (5.00 mL/kg) [39]. Another one hour after ethanol instillation, the animals were sacrificed under anesthesia and stomachs were taken for further analysis. The stomachs of the anesthetized rats in each group were opened along a greater curvature and rinsed with normal saline, and then subjected to a general examination to assess any abnormal lesions. The superficial ulcer areas were measured by image J analyzer software. The gastric ulcer index and the rate of protection of ulceration were calculated the following formula:

\[
\text{Ulcer Index} = \frac{\text{Ulcer pixel amount}}{\text{Total gastric pixel amount}} \times 100\%
\]

\[
\text{Inhibition percentage} = \left(1 - \frac{\text{Ulcer Index}_{\text{Moeld}} - \text{Ulcer Index}_{\text{treated}}}{\text{Ulcer Index}_{\text{Moeld}}}ight) \times 100\%
\]

**Measurement of related biochemical indexes in gastric tissues**

Tissue samples of the stomachs were weighed, minced, and homogenized with cold PBS buffer (pH=7.4, v/v=1:9), a portion of each stomach tissue (0.5 g) was cut into small pieces and 4.5 mL of cold PBS were added. Additionally, the mixture was homogenized on ice with a handheld homogenizer (S10, SCIENTZ, Ningbo). Some tissue homogenates were centrifuged for 2500 rpm at 4 °C for 10 min, and then the supernatants were used to determine the levels of SOD, CAT, GSH and GSH-Px, respectively. Moreover, the other portion was centrifuged for 10 min at 5000 rpm to determine the level of TNF-α and IL-6 were analyzed by enzyme immunoassays.

**Gastric histopathological assessments**

The ulcerated stomachs were washed twice in ice-cold saline, and approximated regions of individual stomach (between cardiac and pylorus, the fundus) were sampled and cross-trimmed based on the lumen. Furthermore, all trimmed fundi were fixed in 10% (V/V) neutral buffered formalin for 24h, and paraffin sections were then cut to a thickness of 5 μm and stained with hematoxylin and eosin (H&E) for histological evaluation according to standard procedures.

**Statistical analysis**
All data was analyzed by the statistical package of social sciences (SPSS, version 20.0). The data with normally distribution and homogeneous variances were expressed as the mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance. Differences were considered significant at values of $P < 0.05$.

**Ethical approval**

Experimental protocols used in the present study were approved by the Animal Experimental Ethics Committee of Beijing University of Chinese Medicine (Beijing, China).

**Declarations**

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This research received no external funding.

**Conflicts of interest**

The authors declare that they have no conflicts interests.

**Availability of Data and Materials**

The data generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

**Authors’ contributions**

Experiments were designed by YZ and HK and conducted by JL, JH, MLZ and JSW. Data was analyzed by YZ, JJC and HHQ. Manuscript was prepared by JL and JH. All authors read and approved the final manuscript.

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