Supporting Information

An Exploration of Glutaredoxin 1 Oxidative Modification in Carbon Nanomaterials Induced-Hepatotoxicity

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Experimental methods

Chemicals: Graphene oxide nanosheets were obtained from JCNO (Nanjing, China). Carbon nanotubes were from Chengdu Organic Chemicals Co., Ltd (Chengdu, China). $C_6H_8O_7$, Urea, DMF, H_2SO_4, HNO_3, SDS, CH_3OH were purchased from Sinopharm Chemical Reagent. Co. Ltd. (Shanghai, China). Paraformaldehyde was obtained from Solarbio (Beijing, China). Cell Counting Kit-8, Reactive Oxygen Species Assay Kit, Hydrogen Peroxide Assay Kit, Hydroxyl Radical Assay Kit, LDH Cytotoxicity Assay Kit, Nuclear and Cytoplasmic Protein Extraction Kit, DAPI staining solution, the enhanced chemiluminescence (ECL) substrate, β-actin, polyvinylidene fluoride (PVDF) membrane and Annexin V-FITC Apoptosis Detection Kit were purchased from Beyotime Biotechnology (Shanghai, China). Recombinant human Glutaredoxin was from KANGLANG Biotechnology Co., Ltd. (Shanghai, China). Anti-ASK1 antibody, anti-p-ASK1 antibody, anti-JNK antibody, anti-p-JNK antibody, anti-p38 antibody, anti-p-p38 antibody were obtained from Abcam (Burlingame, CA, USA). Anti-GLRX1 antibody were from BOSTER Biological Technology Co., Ltd. (Wuhan,China).

Synthesis of CDs: CDs were synthesized via a simple hydrothermal method. In brief, urea (4 g) and citric acid (2 g) were treated at 160 °C for 10 h in 20 mL of N, N-dimethylformamide (DMF) under sol-vothermal conditions, and the resulting mixture was cooled to room temperature. Then the solution was stirred with 40 mL of NaOH (50 mg/mL) for 10 min at room temperature and then centrifuged at 16000 rpm for 15 min. After washing twice, the HCl aqueous solution (5 wt%) was used to neutralize the CDs. In order to further purify the CDs, the solution was washed with water twice

and resuspended with water.

Cutting CNTs: The chemical method of liquid phase oxidation was used to cut carbon nanotubes. 0.5 g of CNTs were placed in a mixture solution consisted of 60 mL of concentrated sulfuric acid and 20 mL of concentrated nitric acid. And then, the mixture was ultrasonically shaken for 6 h at 40 ℃. Remove the mixed acid from the upper layer by centrifugation and wash with water until neutral. After that, the uncut CNTs were filtered by a 0.4 μm polycarbonate membrane filter. Finally, black carbon nanotube powder was obtained after 72 h freeze drying.

Characterization: CCK-8 assay was performed on a microplate reader (Synergy 2, BioTek, USA). TEM imaging was accomplished by a transmission electron microscope (HT7700 electron microscope, Japan). High-resolution transmission electron microscopy (HRTEM) was performed on a JEM-2100 electron microscope (JEOL Ltd., Japan). Dynamic light scattering (DLS) was recorded on a Malvern zeta sizer Nano-ZS90 (Malvern Instruments, UK). Intracellular ROS detection were performed by the ImageStreamX multispectral imaging flow cytometer (USA). Confocal fluorescence imaging was performed on a TCS SP8 confocal laser scanning microscopy (Leica Co., Ltd. Germany). Proteomic analysis was performed by Q Exactive (Thermo Fisher) through LC-MS/MS. Gel electrophoresis was run using the MiniPROTEAN Tetra cell and the band was imaged by ChemiDoc™ Touch Imaging system (Bio-Rad, Hercules, CA, USA).

Experimental details

Cell Cultures: Human normal liver cell line (HL7702) cells were purchased from Procell

Life Science&Technology Co., Ltd. HL-7702 were cultured in media consisting of DMEM with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin.

Cell Cytotoxicity Assay: CNMs-induced cytotoxicity in HL-7702 cells were assessed by Cell Counting Kit-8 (CCK-8) assay. HL-7702 cells were firstly seeded into a 96-well plate at the density of 5000 cells per well for 24 h. The cells were then incubated respectively with different concentrations (100, 200, 400 μg/mL) and morphologies (GO, CNTs, CDs) of CNMs at 37 °C in a 5% CO_2 atmosphere for 24 h. In addition, the cells were incubated with concentration of 400 μg/mL and three types of CNMs at 37°C and 5%CO_2 for 12 and 36 hours. Subsequently, cells were exposed to CCK-8 solution (20 μL per well) for 1 h. Finally, the absorbance at 450 nm was measured using Synergy 2 microplate readers.

Annexin V-FITC/PI Double Staining: To evaluate the CNMs-induced cytotoxicity, Annexin V-FITC/PI double staining was performed. HL-7702 cells were cultured in a 24 well plate at the density of 10^6 cells/well in DMEM containing 10% FBS. And different morphologies (GO, CNTs, CDs) of CNMs were incubated with cells for 24 h. Then, Cells were harvested from the 24 well plate, washed once with cold DMEM, incubated at RT in the dark with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) in Annexin V binding buffer for 20 min. Finally, the cells were analyzed by ImageStream^X Mark II imaging flow cytometer.

Lactate Dehydrogenase (LDH) Release Assay: HL7702 cells were seeded in a 96-well plate and incubated for 24 h. Then, different concentration (100, 200 or 400 μg/mL) of GO, CNTs or CDs were added respectively. Meanwhile, a sample blank control group

and a positive control group with LDH releasing reagent for 1 h were set. After incubating for another 24 h, the cell culture plates were centrifuged for 5 min and 120 μL of supernatant from each well was added to a new 96-well plate. Next, 60 μL of LDH detection working solution was added to each well and incubated for another 30 min, and finally, the absorbance at 490 nm of each well was monitored by Synergy 2 microplate readers.

Intracellular ROS Detection: The fluorescence probe DCFH-DA was used to assess the oxidative stress caused by CNMs. Briefly, the HL-7702 cells were seeded in 6-well plates for 24 h. Then different morphologies (GO, CNTs, CDs) of CNMs were added and incubated with cells for 24 h. Then the cells were loaded with DCFH-DA fluorescent probe (10 μM) and maintained in a 37 °C incubator gassed with CO_2 (5%) for 30 min. After washing them three times with PBS solutions, the fluorescence of cells was analyzed using ImageStream^X Mark II imaging flow cytometer.

Intracellular H_2O_2 Detection: The Hydrogen Peroxide Assay Kit was used to assess the H_2O_2 level. H_2O_2 oxidizes ferrous ion into ferric ions, and then forms purple products with xylenol orange. The concentration of H_2O_2 can be determined by detecting absorbance. HL-7702 liver cells were incubated respectively with GO (400 μg/mL), CNTs (400 μg/mL) and CDs (400 μg/mL) for 24 h. After washing, digestion and centrifugation, 200 μL of Lysis solution was added to break and lysate cells. After centrifugation at 4 ºC for 5 min (12000 g), supernatant was taken for subsequent determination. All of this was done on ice. Then 50 μL of sample was added into 96-well plate. 100 μL of H_2O_2 detection reagent was added into each well, mixed gently

by shaking, and left at room temperature for 30 min. Finally, the absorbance at 450 nm was measured using Synergy 2 microplate readers.

Intracellular •OH Detection: The release of •OH under the stimulation of CNMs with different morphologies was detected by improved Griess colorimetry. In simple terms, the method uses nitroimidazole to react with •OH to produce nitrite, which is then detected by the Griess method. The colorless Griess solution reacts with nitrite ions in a concentration-dependent manner and turns red. HL-7702 cells were incubated respectively with GO (400 μg/mL), CNTs (400 μg/mL) and CDs (400 μg/mL) for 24 h, and homogenated under an ice bath. The centrifugally separated cytoplasm was analyzed by Griess method, and the absorbance at 450 nm was measured by Synergy 2 microplate readers.

DAPI Staining: HL7702 were seeded in 20-mm glass bottom dishes and cultured for 24 h. And then, GO (100, 200, 400 μg/mL), CNTs (400 μg/mL) and CDs (400 μg/mL) were added respectively and cultured for another 24 h. Finally, all the cells were incubated with DAPI dye for 5 min in the dark at 37 °C. Fluorescence images were then collected by Leica TCS SP8 confocal laser scanning microscope.

LC-MS/MS Assay: We evaluated the oxidative modification of GLRX1 using LC-MS/MS assay. GLRX1 was incubated with •OH (200 μM) or H_2O_2 (1 mM) separately at 37 °C for 2 h. Then the proteins were exposed to trypsin digestion and the polypeptides were separated from hydrolysates using a C-18 extraction column. Finally, proteomic analysis was performed to assess the oxidative modifications of GLRX1 at the cysteine thiol groups via LC-MS/MS assay.

BCA Assay: Protein concentrations are determined using the Enhanced BCA Protein Assay Kit. Protein samples extracted from each group and standard protein sample were added to a 96-well plate. Then, 200 μL of BCA working solution were added to each well at 37 ºC for 20 min. Finally, the absorbance at 562 nm was measured by a Synergy 2 microplate reader.

Protein Extraction: HL-7702 cells were incubated with GO, GO with CAT, GO with SOD, •OH or H_2O_2 for 24 h. After washing once with PBS, the cells were lysed with RIPA lysate buffer (containing protease inhibitor PMSF) at 4 °C for 30 min. Then the lysed samples were centrifuged at 4 °C for 10 min (12000 rpm). Finally, the extracted proteins were stored at 80 °C for later use.

Western Blot Assay: The equivalent protein samples (20 μg, quantitative calculation by Enhanced BCA Protein Assay Kit in Figure S8) were separated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE), and then the target proteins were transferred to the polyvinylidene fluoride (PVDF) membranes (100 V, 1 h). After blocking for ten minutes, the PVDF film was incubated with primary antibody (1:1000) overnight at 4 °C. Next, the PVDF film continued to be incubated at room temperature for 1.5 h and then washed with TBST buffer for 30 min, and incubated with the horseradish peroxidase labeled secondary second antibodies under shaking for 1 h. Finally, the enhanced chemiluminescence reagent was added and incubated for 3 min, and ChemiDoc™ Touch imaging system (Bio-RAD, USA) was used for imaging. The tested proteins were GLRX1, p-ASK1, ASK1, p-JNK, JNK, p-p38, p38 and β-actin.

Supporting Figures

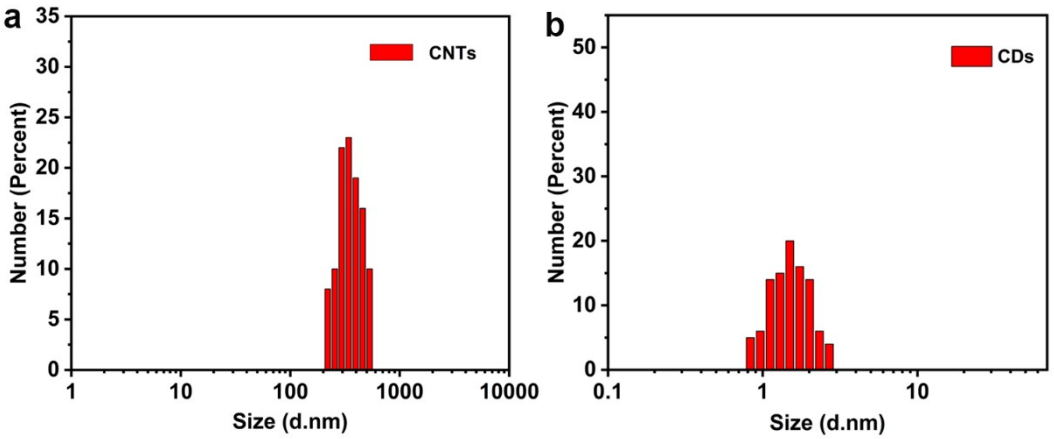

Fig. S1 Particle size distributions of (a) CNTs and (b) CDs monitored using the dynamic light scattering (DLS) technique.

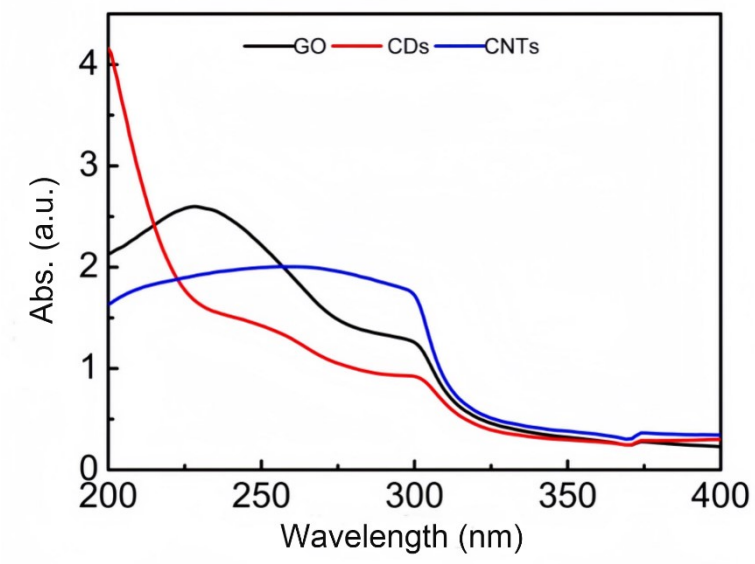

Fig. S2 UV-VIS spectroscopy of three types of GO, CNTs, CDs.

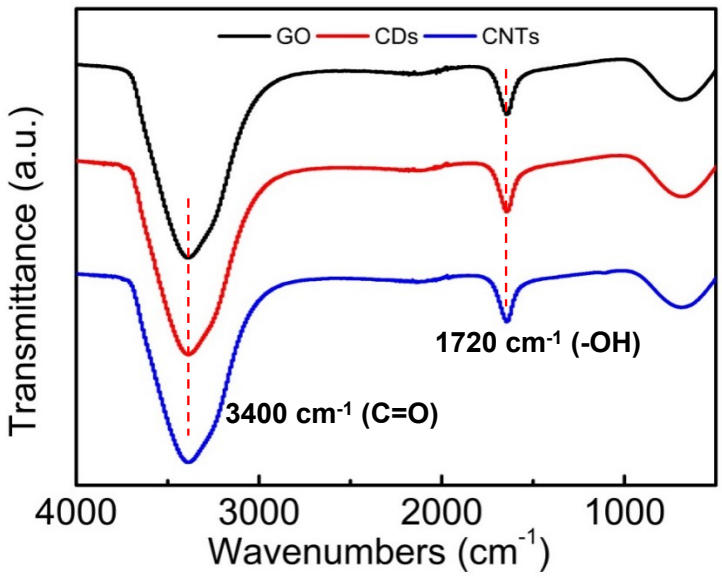

Fig. S3 FT-IR spectra of three types of GO, CNTs, CDs.

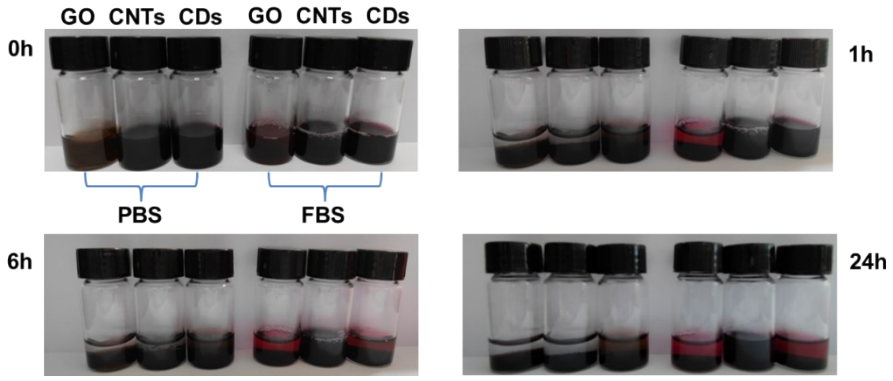

Fig. S4 Optical photograph showing the solution stabilities of CNMs in PBS and FBS (DMEM cell culture medium containing 10% FBS) for different time.

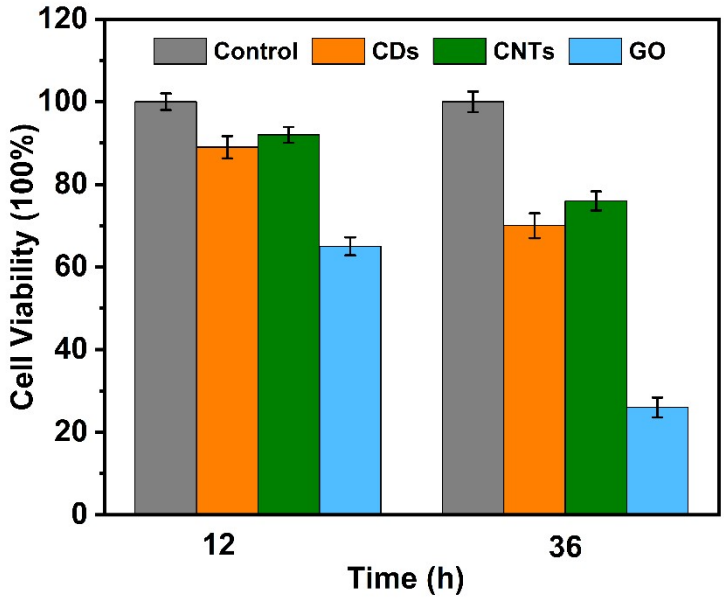

Fig. S5 Time-dependent cytotoxicity by incubating the HL-7702 cells with 400 μg/mL of GO, CNTs or CDs for 12 h and 36 h, respectively.

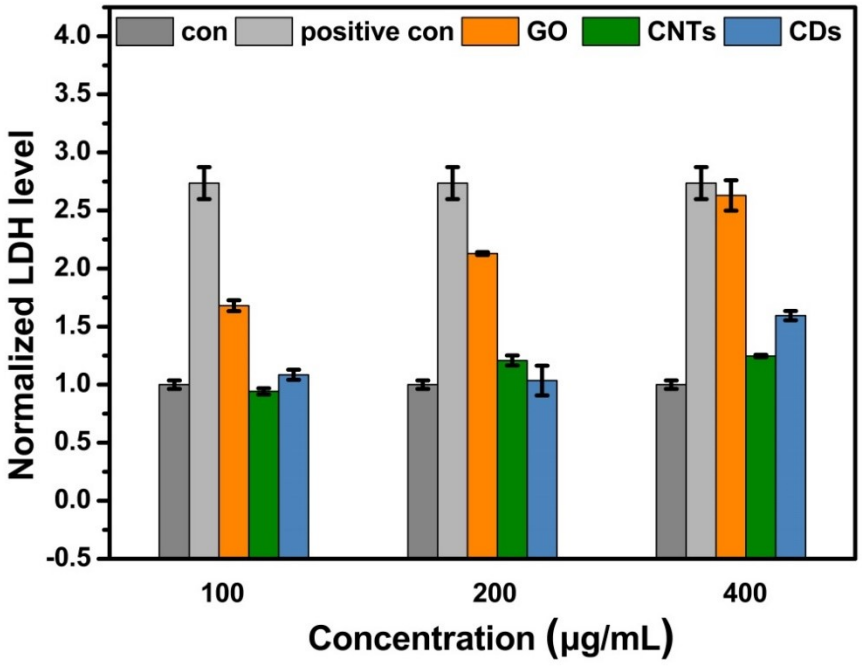

Fig. S6 Level of released LDH after incubating cells with different concentration of CNMs for 24 h. Data are shown as mean ± SD of three independent experiments performed in duplicate.

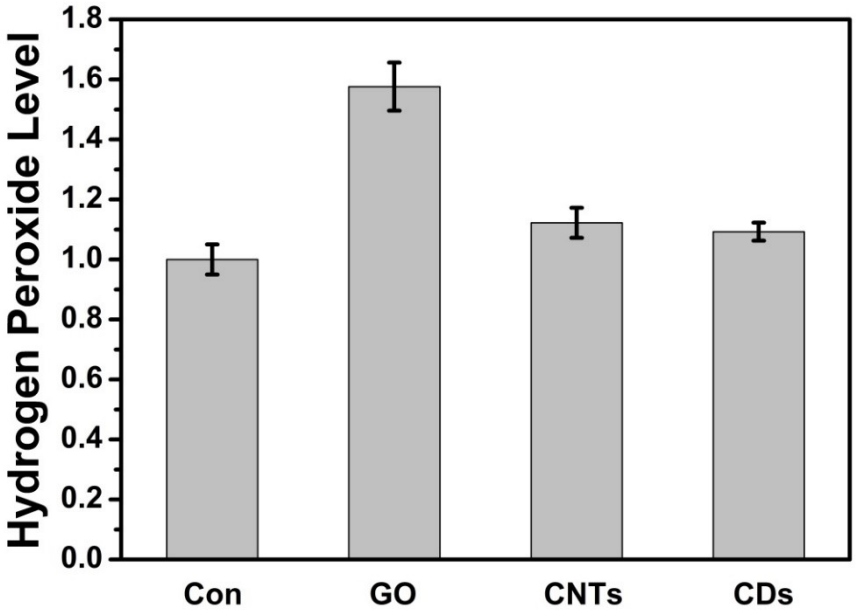

Fig. S7 H_2O_2 level in different CNMs (400 μg/mL)-administered cells for 24 h. Data are shown as mean ±S.D. of three independent experiments performed in duplicate.

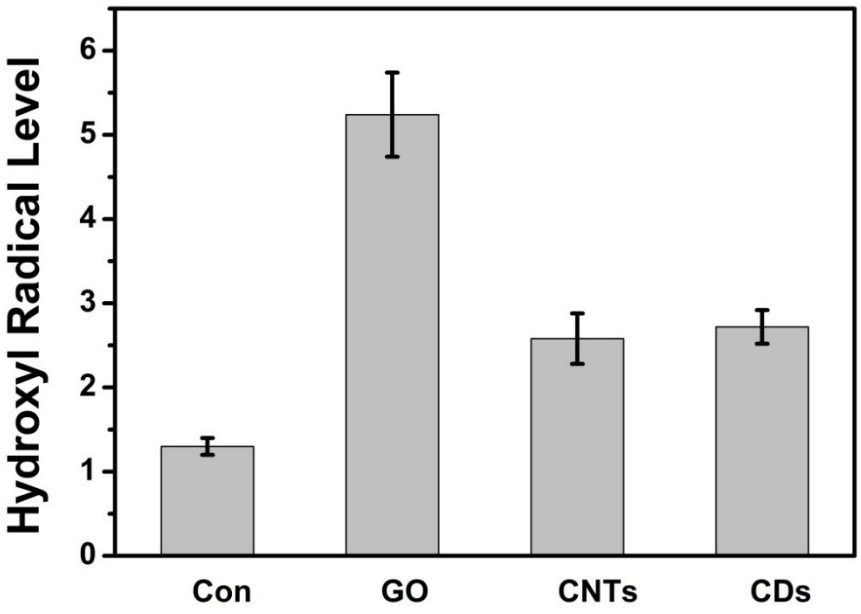

Fig. S8 •OH level in different CNMs (400 μg/mL)-administered cells for 24 h. Data are shown as mean ±S.D. of three independent experiments performed in duplicate.

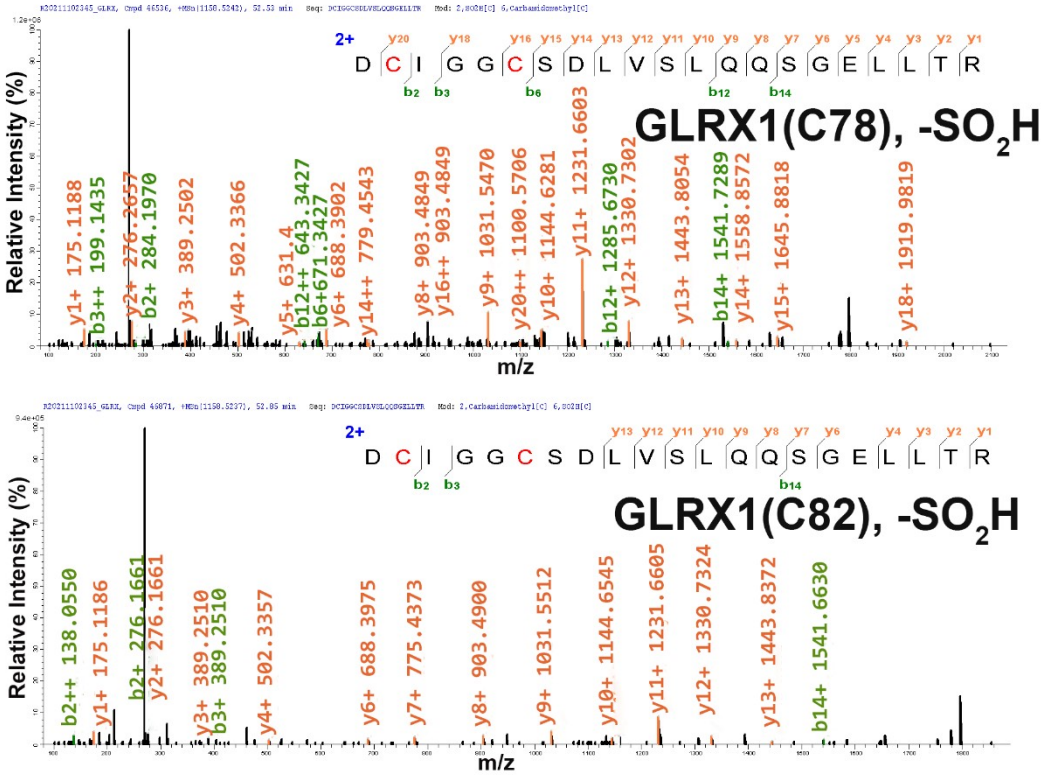

Fig. S9 LC–MS/MS analysis of the oxidative modifications at the active sites of GLRX1 by H_2O_2 (200 μM).

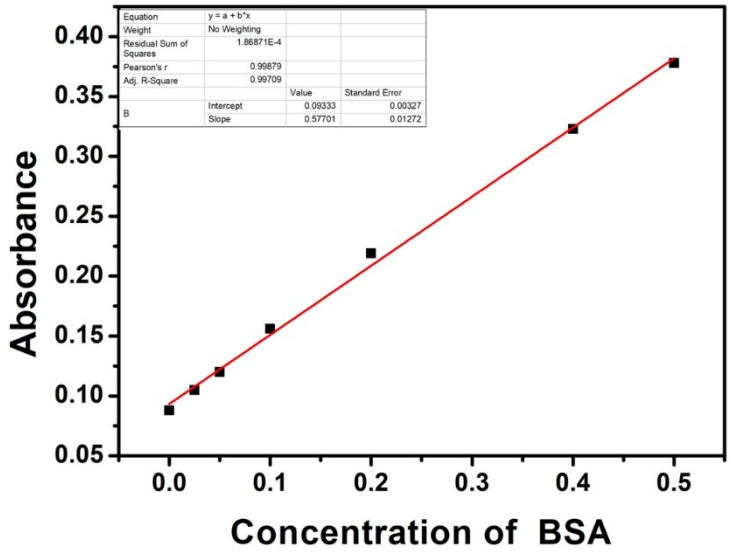

Fig. S10 The bicinchoninic acid (BCA) assay for protein quantitation.

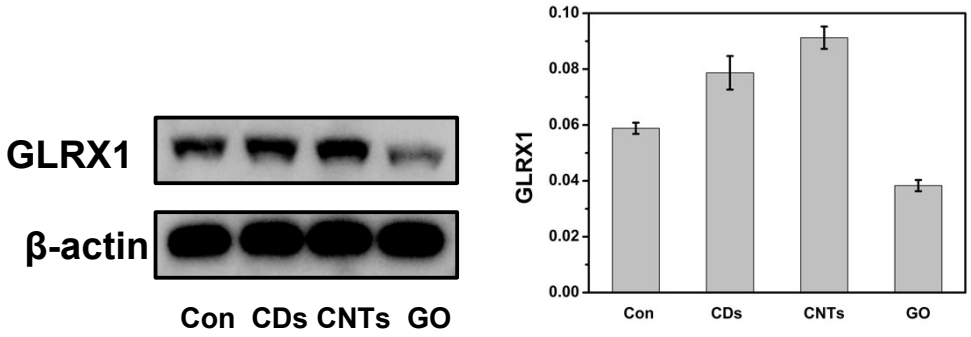

Fig. S11 Western blot analysis of GLRX1 in the HL7702 human liver cells exposed to 400 μg/mL of CDs, CNTs or GO.