

Supporting Information

An Ultrasound Activated Oxygen Generation Nanosystem Specifically Alleviates Myocardial Hypoxemia and Promotes Cell Survival Following Acute Myocardial Infarction

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Methods

Materials. Hydrochloric acid solution (HCl, 37%), ammonia aqueous solution ($\text{NH}_3 \cdot \text{H}_2\text{O}$, 25 wt%), cetyltrimethylammonium bromide (CTAB), calcium chloride (CaCl_2), hydrogen peroxide (H_2O_2 , 30%), tetraethyl orthosilicate (TEOS), absolute ethanol were purchased from Sinopharm Chemical Reagent Co., Ltd. Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) dichloride complex ($[\text{Ru}(\text{dpp})_3]\text{Cl}_2$), heneicosane (HE), 3-aminopropyltriethoxysilane (APTES), were from Energy Chemical. Simulated body fluid (SBF), cell counting kit-8 (CCK-8), DMEM medium, phosphate buffered saline (PBS, pH 7.4), fluorescein isothiocyanate (FITC), and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Beyotime Biotechnology. 0.05% Trypsin-EDTA, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), and penicillin-streptomycin solution were obtained from Gibco. Calcein AM/propidium iodide (PI) double staining kit were from Dojindo Molecular Technologies. Methoxy PEG silane was from Sigma-Aldrich. All chemicals were used as received without further purification.

Synthesis of CMHP. The MSN were synthesized as described and redispersed into water. Then, 10 mL of the mixture composed of CaCl_2 (100 mM) and H_2O_2 (30%) were added into the MSN dispersion in a stirring manner under cooling conditions. After 2 h, ammonia solution (5 wt%) was added into the above suspension in a dropwise manner under cooling conditions. The reaction was ended after 12 h and the suspension was allowed to stay overnight. Then the CaO_2 -encapsulated MSN (denoted as $\text{CaO}_2@\text{MSN}$) were collected by centrifugation and wash repeatedly, followed by air drying. Afterwards, 10 mg of $\text{CaO}_2@\text{MSN}$ were redispersed in 5 mL of HE chloroform solution. After continuous stirring for 12 h, the suspension was centrifuged and washed to produce HE-loaded nanocomposites (denoted as $\text{CaO}_2@\text{MSN-HE}$). Finally, $\text{CaO}_2@\text{MSN-HE}$ (5 mg) were dispersed into methoxy PEG silane ethanol solution (0.2 mg ml^{-1}) and stirred for another 24 h. Residual PEG were rinsed with ethanol to produce PEGylated $\text{CaO}_2@\text{MSN-HE}$ (denoted as $\text{CaO}_2@\text{MSN-HE-PEG}$, abbreviated as CMHP).

Evaluation of the loading amount of CaO_2 in the CMHP. The amount of CaO_2 loaded in the nanosystem was determined by analyzing the change of UV-vis absorbance of potassium permanganate (KMnO_4) at 525 nm. The UV-vis absorbance of KMnO_4 changes with the redox

reaction with CaO₂ in acidic media. The measurement process was performed on a shaking table at a shaking speed of 100 rpm. Typically, the nanosystem (1.0 mg) were encapsulated into a dialysis bag and immersed into acidic deionized water (containing HCl, 2.0 M) at 45 °C. Immediately, manganese sulfate solution (50 mM) was added to the dispersion solution, followed by adding of KMnO₄ standard solution dropwise. The color change was observed and the absorption spectrum of KMnO₄ solution was recorded by a UV-vis spectrophotometer. The H₂O₂ solution was used as the positive control, which acts as the substrates of the KMnO₄ mediated redox reactions. The relationship between the concentration of H₂O₂ or CaO₂ and the absorbance of KMnO₄ was compared and the amount of CaO₂ in the nanosystem was then calculated.

Characterization. Transmission electron microscopy (TEM) images were recorded on a JEM-2100F electron microscope. N₂ adsorption-desorption analysis was performed on a N₂ adsorption-desorption analyzer (Micrometitics Tristar 3000). Surface area and pore volume were determined by Barrette-Joynere-Halenda (BET) analysis. Powder X-ray diffraction (XRD) patterns were recorded on a Rigaku Ultima IV diffractometer. Thermogravimetry (TG) and differential scanning calorimetry (DSC) were carried out on a thermal analyzer (TA Instruments). Zeta potential was determined on Malvern Zetasizer Nanoseries (Nano ZS90).

Observation and Measurement of Oxygen Release. Deoxygenated PBS (20 mL) at room temperature was filled with nitrogen and sealed. After the injection of CMHP-dispersed PBS (1 mL) followed by intermittent US irradiation (1.0 MHz, 0.6 W cm⁻²), the real-time oxygen concentrations in PBS was recorded by a JPSJ-605F portable dissolved oxygen meter (INESA Scientific Instrument Co., Ltd, China). The DOC in CMHP-dispersed PBS (pH 7.4, 40 °C and 37 °C) were also measured. Additionally, the pH values of the CMHP-dispersed PBS were measured by a PHSJ-4F pH meter (INESA Scientific Instrument Co., Ltd, China).

Cell Culture. Rat ventricular H9c2 cardiomyocyte cells were from cell bank of Chinese Academy of Sciences, and cultured in DMEM medium (pH 7.4) supplemented with 10% FBS at 37 °C in a humidified 5% CO₂ atmosphere (*Normoxia*). To mimic the hypoxic environment, cells were incubated in a humidified 1% O₂/5% CO₂/94% N₂ atmosphere (*Hypoxia*) at 37 °C for 48 h.

***In Vitro* Evaluation of Cell Survival.** Initially, H9c2 cells were seeded in 96-well plates for 24 h at a density of 5×10^3 cells *per* well under normoxia atmospheres (20% O₂/5% CO₂/75% N₂). Then, cells were transferred to hypoxia (1% O₂/5% CO₂/94% N₂) and incubated for another 48 h. H9c2 cells were then incubated with 100 μ L of culture media containing different concentrations of CMHP under hypoxia. After 8 h of incubation, cells were exposed to US irradiation (1.0 MHz, 0.6 W cm⁻²) for 60 s and further incubated for 24 h under hypoxia atmospheres. Then, 10 μ L of CCK-8 was added to each well and further incubated for 1 h. Afterwards, the absorbance of the sample solution was measured at 450 nm using a microplate reader (Bio-Tek ELx800, USA). Complete growth media without samples were used as the negative controls. The sonotoxicity of US irradiation was also evaluated by treating cells on complete growth media with US irradiation (1.0 MHz, 0.6 W cm⁻²) for 60 s. Data are represented as means \pm SD (n = 6).

Calcein-AM/PI staining were performed. Briefly, after discarding the culture media and rinsing cells with PBS for several time, cells were incubated 100 μ L of calcein-AM solution and 100 μ L of PI solution. After incubation for 30 min, cells were rinsed by PBS twice and visualized by inverted fluorescence microscopy (Olympus IX71, Japan).

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured by using JC-1 fluorescence probe. In brief, H9ce cells were seeded in 6 well plates at a density of 1×10^5 cells per well and incubated overnight. Then, cells were incubated with culture media containing CMHP for 6 h, followed by US irradiation. After incubation for another 12 h, cells were rinsed and incubated with JC-1 dye (2 μ M) for 20 min at 37 °C. Afterwards, cells were rinsed and observed by confocal laser scanning microscopy (CLSM).

The ATP level was measured by the ATP bioluminescence assay kit (Roche, Switzerland). Briefly, cells were homogenized in lysis buffer and centrifuged at 15000 rpm for 15 min at low temperature. The supernatants were collected and the ATP level was determined by a luminescence plate reader (Biotek, USA).

The activity of cytochrome C oxidase was determined according to the manual of the cytochrome C oxidase activity assay kit (Abcam). Typically, the enzyme dilution buffer was mixed

with the mitochondrial fractions, followed by addition of the ferrocytochrome c substrate solution. The change in absorbance at 550 nm was recorded with a 5 s delay and 10 s interval for 6 readings on a microplate reader (Biotek, USA).

Intracellular ROS level was investigated as follows. H9c2 cells were incubated for 48 h under hypoxia at 37 °C. Then, cells were co-incubated with CMHP for 6 h, followed by US irradiation (1.0 MHz, 0.6 W cm⁻², 60 s). After incubation for another 12 h under hypoxia, cells were rinsed and treated with DCFH-DA (100 µL, 10 µM) for 30 min at 37 °C. The fluorescence was observed by fluorescence microscopy (Olympus IX71, Japan). H9c2 cells treated with fresh CMHP or US irradiation alone was also measured. Cells treated with fresh culture media under hypoxia were used as the control group.

***In Vitro* Detection of Cellular Hypoxia by Optical Probe.** H9c2 cells were cultured under hypoxia at 37 °C for 48 h. Then, cells were co-incubated with [Ru(dpp)₃]Cl₂ (10 µg mL⁻¹) for 4 h, followed by washing with PBS for several times. Cells were then incubated with CMHP for 4 h, followed by US irradiation (1.0 MHz, 0.6 W cm⁻², 60 s). After incubation for another 8 h, the intracellular oxygen level was measured by detecting the luminescence intensity of [Ru(dpp)₃]Cl₂ ($\lambda_{\text{ex}} = 450 \text{ nm}$, $\lambda_{\text{em}} = 600 \text{ nm}$). Cells treated with fresh culture media under hypoxia were used as the negative control. Cells cultured in a humidified 5% CO₂ atmosphere (normoxia) at 37 °C for 48 h were used as the positive control.

Animal Experiments. Male Sprague Dawley (SD) rats weighted 200-250 g were purchased from Shanghai Super - B&K laboratory animal Corp. Ltd. All animal procedures were performed under the protocols approved by the Institutional Animal Care and Use Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. All animal experiments were carried out in agreement with the guidelines of Shanghai Lab. Animal Research Center (animal ethical approval No. HKDL [2018]323). AMI model was established by ligating the left anterior descending (LAD) coronary artery of SD rats with 4-0 polypropylene sutures under 2.5% isoflurane anaesthesia according to reported methods in literatures.^[1]

***In Vivo* Evaluation of US-Activated Oxygen Release on AMI of Rats.** Male SD rats were randomly divided into five groups: Sham group (underwent thoracotomy without LAD ligation);

MI group (injection with saline); US group (injection with saline followed by US irradiation); CMHP group (injection with CMHP only); CMHP + US group (injection with CMHP followed by US irradiation). The treatments (injection or US irradiation) were given at 3 days post MI to investigate their short- and long-term effects on AMI. To guarantee a more even distribution of nanomaterials in the myocardium, the CMHP-dispersed saline (4.0 mg mL⁻¹) was injected into the center of the infarction as well as the apical, proximal, lateral, and septal wall regions bordering the infarcted rat heart (50 µL, 10 µL per region), followed by US irradiation (1.0 MHz, 0.6 W cm⁻²) for 30 s. The temperature at infarcted heart of rats after US irradiation was recorded by an infrared thermal imaging system (FLIR Systems Inc., USA).

The infarcted myocardium was analyzed by echocardiography (Vevo 2100, VisualSonics, Canada) equipped with a 21 MHz transducer at 4 weeks post treatment. Typically, after shaving in the anterior and left lateral thoracic regions, rats were placed in the supine position. Afterwards, the thoracic regions were smeared with ultrasound transonic gel to optimize visibility. Both conventional two-dimensional and M-mode echocardiographic images in a parasternal short and long axis were then acquired by echocardiography. Off-line imaging measurements were performed by one observer blind to the animal's condition. The left ventricular posterior wall (LVPW) thickness and the left ventricular septum (LVS) thickness as well as the left ventricular internal dimensions during systole (LVIDs) and diastole (LVIDd) were measured according to the American Society of Echocardiography technique.^[3] The value of each rat was the mean of three measurements. Rats with infarction sizes smaller than 25% of the left ventricle free wall were excluded from the study. The left ventricular ejection fraction (LVEF) and fractional shortening (FS) were then calculated according to the following equation 1 and 2:

$$LVEF = \frac{LVEDV - LVESV}{LVEDV} \times 100\% \quad (1)$$

$$FS = \frac{LVIDd - LVIDs}{LVIDd} \times 100\% \quad (2)$$

Here, the LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV) were estimated according to the following equation 3 and 4:

$$LVEDV = \frac{7.0}{2.4 + LVIDd} \times LVIDd^3 \quad (3)$$

$$LVESV = \frac{7.0}{2.4 + LVIDs} \times LVIDs^3 \quad (4)$$

The body weight of rats were recorded every four days for 4 weeks. At the end of the evaluation period of 4 weeks, rats were sacrificed and the infarcted hearts at the level of injection site were chosen for histopathological analyses (H&E staining, 2,3,5-triphenyltetrazolium chloride (TTC) staining, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL), and Ki67 immunofluorescence staining). Analysis of MI size by echocardiography was performed on 52 surviving rats. MI size (% of left ventricle perimeter) was determined as the average of six sections sampled at 2-mm intervals from the apex based on the equation 5.^[2] The infarct size for each plane was the mean of three measurements and the final value was the mean of all three planes.

$$\text{Infarct Size} = \frac{\text{coronal infarct perimeter (epicardial + endocardial)}}{\text{total coronal perimeter (epicardial + endocardial)}} \times 100\% \quad (5)$$

Besides, the main organs of liver, spleen, lung, and kidney of rats were harvested for H&E staining. The Si elements in these organs were measured by inductively coupled plasma optical emission spectrometer (ICP-OES, Agilent 700 Series, USA). Briefly, the tissues of liver, spleen, lung, and kidney in all groups were dried, acidolysis, homogenized, and analyzed by the ICP-OES.

Statistics. Results were presented as mean \pm standard deviation (SD). Statistical comparisons were performed with student's *t*-test and 1-way ANOVA. $p < 0.05$ was considered statistically significant.

References.

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- [2] S. G. Ong, B. C. Huber, W. H. Lee, K. Kodo, A. D. Ebert, Y. Ma, P. K. Nguyen, S. Diecke, W. Y. Chen and J. C. Wu, *Circulation*, 2015, **132**, 762.
- [3] R. M. Lang, L. P. Badano, V. Mor-Avi, J. Afilalo, A. Armstrong, L. Ernande, F. A. Flachskampf, E. Foster, S. A. Goldstein, T. Kuznetsova, P. Lancellotti, D. Muraru, M. H. Picard, E. R. Rietzschel, L. Rudski, K. T. Spencer, W. Tsang and J. U. Voigt, *J. Am. Soc. Echocardiog.*, 2015, **28**, 1-39.e14.

Equation S1. The reaction of CaCl_2 with H_2O_2 under mildly alkaline conditions (ammonia solution) leads to the generation of CaO_2 species.

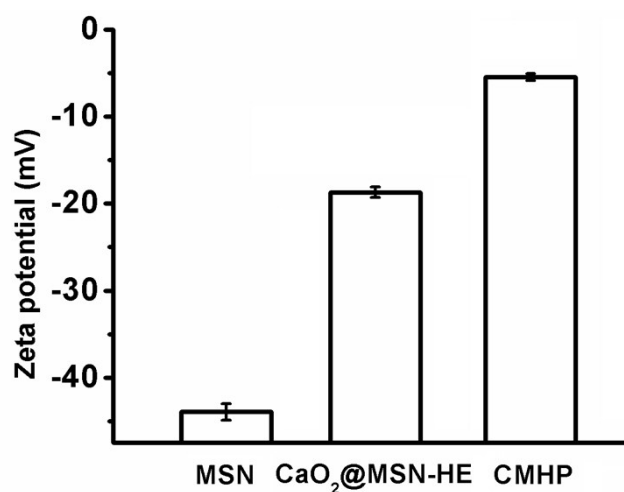
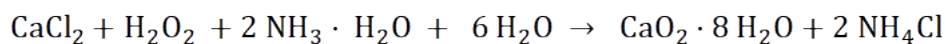


Fig. S1 Zeta potentials of MSN, CaO_2 @MSN-HE, and CMHP. The serial changes in zeta potentials demonstrate successful conjugation of HE and PEG.

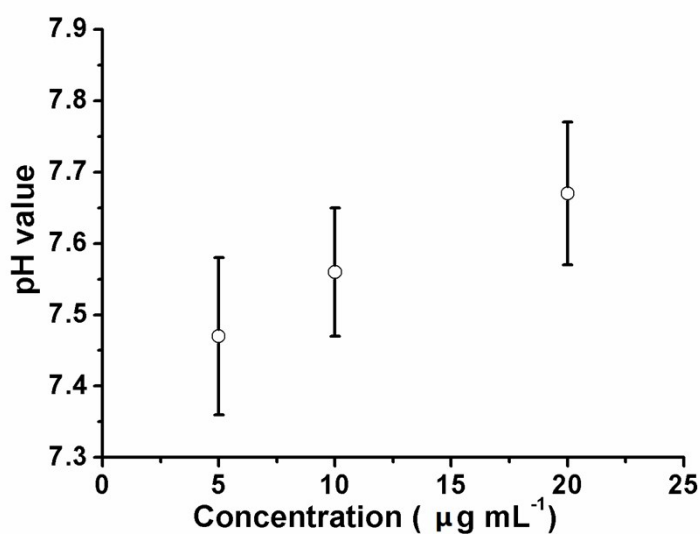


Fig. S2 pH value variation in CMHP-dispersed PBS media.

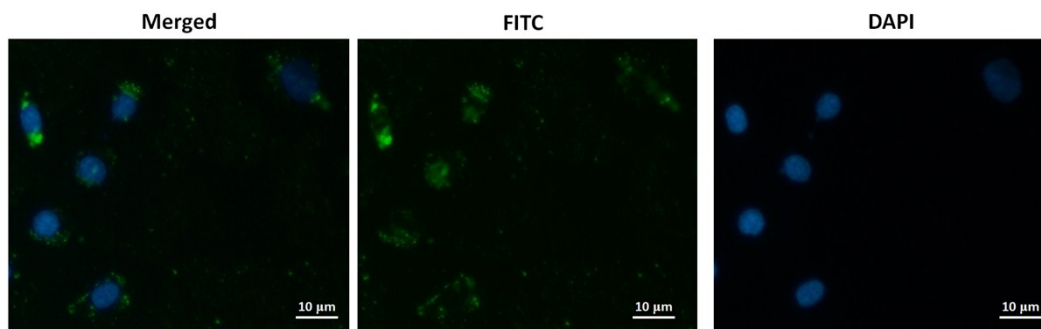


Fig. S3 Cellular uptake of FITC-labeled CMHP. The cell nuclei were stained with DAPI (blue).

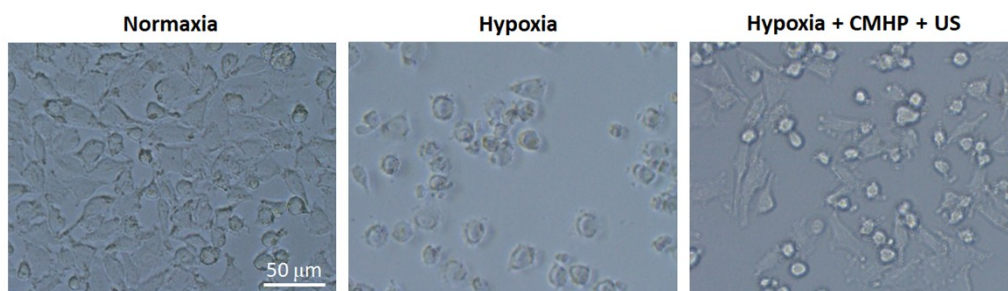


Fig. S4 The cell morphology after incubation under normal oxygen and hypoxic conditions at 24 h post different treatments.

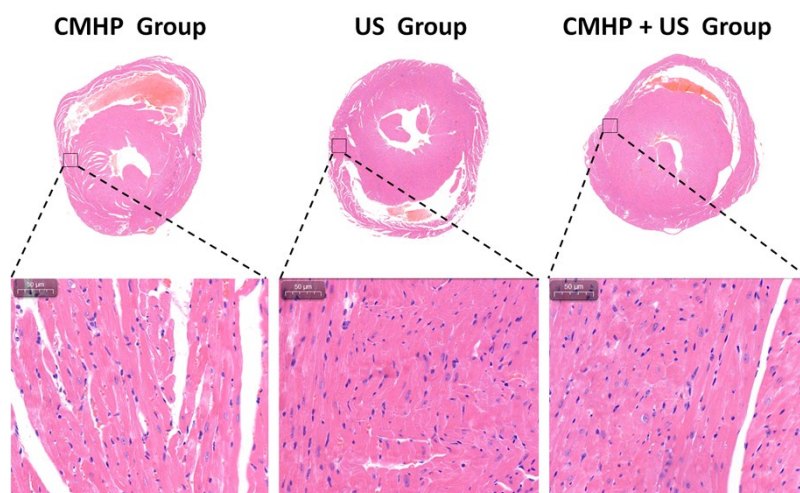


Fig. S5 H&E staining of hearts of male SD mice with healthy hearts 4 weeks post treatments with CMHP nanomaterials and/or US irradiation. Scale bars: 50 μ m. No obvious pathological changes of heart was observed in each group, indicating the good biocompatibility of the US activatable oxygen generation nanosystem.

Table S1 The survival of rats at different times in each group.

Group	Death at 24 h post ligation	Death at 3 d post ligation	Death at 15 d post ligation	Death at 28 d post ligation
Sham (n = 10)	0	0	0	0
MI (n = 20)	2	3	4	2
US (n = 20)	2	2	6	1
CMHP (n = 20)	1	3	4	1
CMHP + US (n = 20)	2	1	3	0

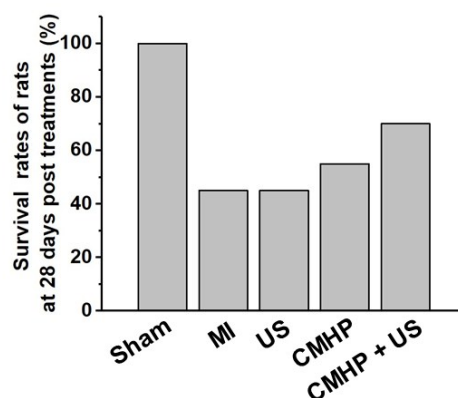


Fig. S6 The survival rates of rats in each group at 4 weeks post different treatments.

Table S2 The heart rate of rats at different times in each group calculated based on the electrocardiogram.

Time	Sham	MI	US	CMHP	CMHP + US
Pre-operation	365.6 ± 40.2	377.6 ± 25.2	375.4 ± 38.5	383.6 ± 31.2	381.2 ± 37.8
3 days Post-operation	353.6 ± 25.5	393.3 ± 30.7	403.6 ± 35.2	435.8 ± 24.1	393.7 ± 39.1
14 days Post-operation	373.6 ± 23.0	438.1 ± 29.3	408.6 ± 41.9	418.1 ± 31.3	391.2 ± 33.2
28 days Post-operation	375.1 ± 29.1	421.6 ± 40.0 ^a	442.2 ± 30.4	419.0 ± 35.3	378.4 ± 20.2 ^b

^a * $p < 0.05$, in comparison with the Sham group. ^b $p > 0.05$, in comparison with the Sham group.

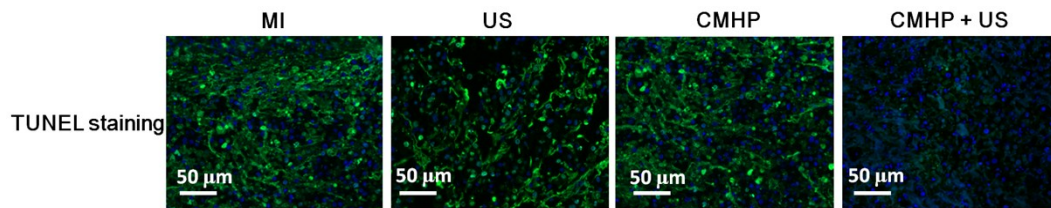


Fig. S7 Representative histological images of TUNEL immunofluorescence stained sections of infarcted myocardial tissues at 4 weeks post different treatments.

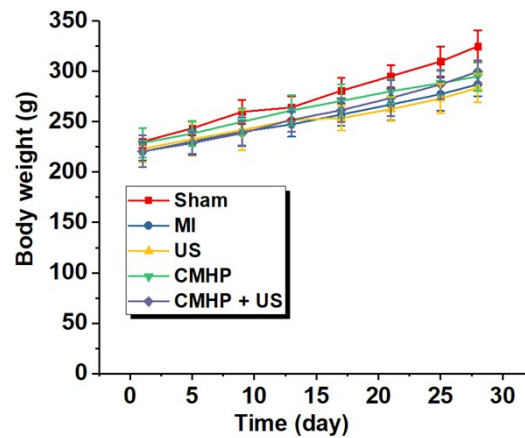


Figure S8. The changes of body weight of male SD rats during 4 weeks feeding in each group.

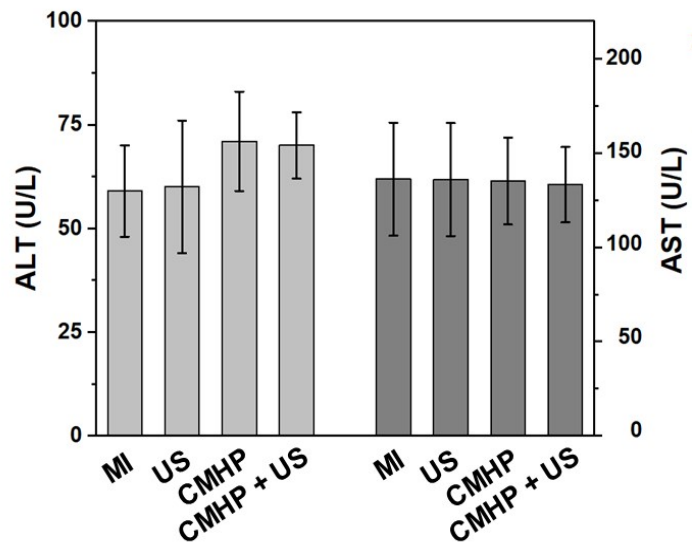


Figure S9. Serum ALT and AST levels of rats in different groups at 4 weeks post treatments.